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# (54) BROAD SPECTRUM INSECTICIDAL POLYPEPTIDE AGAINST LEPIDOPTERAN

PESTS AND METHODS OF USE THEREOF

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- (52) **U.S. Cl.**

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None

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# (57) ABSTRACT

The disclosure provides nucleic acids, and variants and fragments thereof, obtained from strains of *Bacillus thuringiensis* encoding polypeptides having pesticidal activity against insect pests, including Lepidoptera. Particular embodiments of the disclosure provide isolated nucleic acids encoding pesticidal proteins, pesticidal compositions, DNA constructs, and transformed microorganisms and plants comprising a nucleic acid of the embodiments. These compositions find use in methods for controlling pests, especially plant pests.

# 26 Claims, 2 Drawing Sheets

Specification includes a Sequence Listing.

<sup>\*</sup> cited by examiner

# Fig. 1A

Cry1Ea MP372	(1) (1)	1 50  MEIVNNQNQC%PYNCLNNPENEIL®IERSNSTVATNIALE SRLLASATP  -MDNNNQNQC%PYNCLSNPELEIL®IERSNNTVVEDITLG SRLLVSAIP
Cry1Ea MP372	(51) (50)	51 100 IG <mark>GI</mark> ILGLFD <mark>A</mark> IWGSIGPSQWDIFLEQIELLIDQRIEEFARNQAISRLEG IGDFILGLFD <mark>V</mark> IWGAIGRS <mark>E</mark> WDIFLEQIELLI <mark>G</mark> QRIEEFARNQAISRLEG
Cry1Ea MP372	(101) (100)	101 150  SSLYCIYTEAFREWEADPTNPALKEEMRTQFNDMNSILVTAIPLFSVQN SNLYRIYTNAFREWEADPTNLELKEEMRTQFNDMNSAFTTAIPLFSVRC
Cry1Ea MP372	(151) (150)	151 200 YOWPELSVYVQAANLHLSVLRDVSVFGQAWGFDWATWNRRYDDLTRLIPI YEWPLLSVYVQAANLHLSVLRDVSVFGQRWGFDWATWNRRYDDLTTNIGD
Cry1Ea MP372	(201) (200)	201 250 YTDYAKRWYNTGLDRLPRTGGLRNWARFNQFRRELTISVLDIISFFRNYD YTDYAKSWYNTGLNRLPRNDGLRCWARFNRFRRELTISVLDIISFFQNYD
Cry1Ea MP372	(251) (250)	251 <b>V</b> 300  SRLYPIPTSSQLTREVYTDPVINITDYRVGPSFENIENSAIRSPHLMDFL  SRLYPIPTTSQLTREVYTDPVINITDYRVTPSFESIENSAIRSPHLMDFL
Cry1Ea MP372	(301) (300)	301 350 NNXTIDTDLIRGVXYWAGHRVTSHFTGSSQVITXPQYGITANAEPRRT TNXIIIDTDLIRGVXYWAGHRINSRFTGXXFPHXITSPQYGITANAEPRRT
Cry1Ea MP372	(349) (350)	351 400 IAP <mark>STFPGIN-</mark> LFYRTLSNPFFRRSINIIPTLGINVVQGVGFIQPNNAEV IAP <mark>GPFQGXPS</mark> LLYRTLSDPFFRRSINIIPTLGINVVQGVGFIQPNNFES
Cry1Ea MP372	(398) (400)	401 450 LYRSRGTVDSLNELPIDGENSLVGYSHRLSHVTLTRSLENTNITSLPTFV LYRRRGTVDSLDELPIDGENPLVGYSHRLSHVTLTRSLENTNITSLPTFV
Cry1Ea MP372	(448) (450)	451 500 WTHHSATNTNTINPD#ITQIPLVK#FRLGGGT#VI#GPGFTGGDILRRNT WTHHSATDTNTIAPD#ITQIPLVK#FNLHSGA#VA#GPGFTGGDILRRTN
Cry1Ea MP372	(498) (500)	501 550 IGEFVSIQVNINSPITQRYRIRFRYASSRDARITVIGGQIRVDMTLEKT VGNFGD RVNITAPISQRYRVRIRYASITNLRFHTSINGRAINQADFPAT
Cry1Ea MP372	(548) (550)	551  MEIGESLTSREFSYTNFSNPFSFRANPDIIRIÆELPIRGGEÆYIDÆIEL MNSGGNLQSGSFRIAGFETPFEFSDALSTFTIÆAFGFSSGNEVYIDRIEF

# Fig. 1B

Cry1Ea MP372	(598) (600)	601 650  LADATFEEEYDLERAQKAVNALFTSINQIGLKTDVTDYHIDQVSNLVEC  PARVTFEAEYDLERAQKAVNALFTSINQIGLKTDVTDYHIDQVSNLVEC
Cry1Ea MP372	(648) (650)	651 700 LSDEFCLDEKRELSEKVKHAKRLSDERNLLQDENFRGINRQEDRGWRGST LSDEFCLDEKRELSEKVKHAKRLSDERNLLQDENFRSINGQLDRGWRGST
Cry1Ea MP372	(698) (700)	701 750 DITIQGGDDVFKENYVTLPGTFDECYPTYLYQKIDESKLKXYTRYELRGY DITIQGGDDVFKENYVTLPGTFDECYPTYLYQKIDESKLKXYTRYELRGY
Cry1Ea MP372	(748) (750)	751 800 IEDSQDLEIYLIRYNAKHETVNVPGTGSLWPLSAQSPIGKCGEPNRCAPH IEDSQDLEIYLIRYNAKHETVNVPGTGSLWPLSIENSIGPCGEPNRCAPH
Cry1Ea MP372	(798) (800)	801 850 LEWNPNLDCSCRDGEKCAHHSHHFSLDIDVGCTDLNEDLGVWVIFKIKTQ LEWNPNLDCSCRDGEKCAHHSHHFSLDIDVGCTDLNEDLGVWVIFKIKTQ
Cry1Ea MP372	(848) (850)	851 900 DG*ARLGNLEFLEENPLLGEALARVKRAEKKWRDKCEKLEWETNIVYKEA DG#ARLGNLEFLEEKPLLGEALARVKRAEKKWRDKREKLEWETNIVYKEA
Cry1Ea MP372	(898) (900)	901 950 KESVDALFVNSQYDRLQADTNIAMIHAADKRVH <b>S</b> IREAYLPELSVIPGVN KESVDALFVNSQYDRLQADTNIAMIHAADKRVH <b>R</b> IREAYLPELSVIPGVN
Cry1Ea MP372	(948) (950)	951 1000 AAIFEELEGRIFTAKSLYDARNVIKNGDFNNGLSCWN KGHVDVEEQNNH AGIFEELEGRIFTAKSLYDARNVIKNGDFNNGL
Cry1Ea MP372	(998) (1000)	1001 1050 RSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIEDNT RSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIEDNT
Cry1Ea MP372	(1048) (1050)	1051 1100 DELKFSNCVEEEVYPNNTVTCNNYTATQEEREGTYTSRNNGYDE YESNS DELKFSNCVEEEVYPNNTVTCNNYTATQEEREGGAYTSRNHGYGKSYESNS
Cry1Ea MP372	(1098) (1100)	1101 1150
Cry1Ea MP372	(1145) (1150)	1151 1177 TDKVW EIGETEGTFIVDSVELLLMEE TDKVW EIGETEGTFIVDSVELLLMEE

# BROAD SPECTRUM INSECTICIDAL POLYPEPTIDE AGAINST LEPIDOPTERAN PESTS AND METHODS OF USE THEREOF

# CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation application of Ser. No. 16/342,343, filed Apr. 16, 2019, which claims priority to U.S. Provisional Application No. 62/410,045, filed Oct. 19, 2016, which is hereby incorporated herein in their entireties by reference.

# REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

A sequence listing having the file name "7157WOPCT\_ SequenceListing.txt" created on Sep. 19, 2017, and having a size of 47 kilobytes is filed in computer readable form concurrently with the specification. The sequence listing is <sup>20</sup> part of the specification and is herein incorporated by reference in its entirety.

### **FIELD**

The present disclosure relates to naturally-occurring and recombinant nucleic acids obtained from novel *Bacillus thuringiensis* genes that encode pesticidal polypeptides having pesticidal activity against insect pests. Compositions and methods of the disclosure utilize the disclosed nucleic acids, <sup>30</sup> and their encoded pesticidal polypeptides, to control plant pests.

## BACKGROUND

Insect pests are a major factor in the loss of the world's agricultural crops. For example, armyworm feeding, black cutworm damage, or European corn borer damage can be economically devastating to agricultural producers. Insect pest-related crop loss from European corn borer attacks on 40 field and sweet corn alone has reached about one billion dollars a year in damage and control expenses.

Traditionally, the primary method for impacting insect pest populations is the application of broad-spectrum chemical insecticides. However, consumers and government regulators alike are becoming increasingly concerned with the environmental hazards associated with the production and use of synthetic chemical pesticides. Because of such concerns, regulators have banned or limited the use of some of the more hazardous pesticides. Thus, there is substantial 50 interest in developing alternative pesticides.

Biological control of insect pests of agricultural significance using a microbial agent, such as fungi, bacteria, or another species of insect affords an environmentally friendly and commercially attractive alternative to synthetic chemical pesticides. Generally speaking, the use of biopesticides presents a lower risk of pollution and environmental hazards, and biopesticides provide greater target specificity than is characteristic of traditional broad-spectrum chemical insecticides. In addition, biopesticides often cost less to 60 produce and thus improve economic yield for a wide variety of crops.

Certain species of microorganisms of the genus *Bacillus* are known to possess pesticidal activity against a broad range of insect pests including Lepidoptera, Diptera, Coleoptera, Hemiptera, and others. *Bacillus thuringiensis* (Bt) and *Bacillus papilliae* are among the most successful bio-

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control agents discovered to date. Insect pathogenicity has also been attributed to strains of *B. larvae*, *B. lentimorbus*, *B. sphaericus* (Harwook, ed., ((1989) *Bacillus* (Plenum Press), 306) and *B. cereus* (WO 96/10083). Pesticidal activity appears to be concentrated in parasporal crystalline protein inclusions, although pesticidal proteins have also been isolated from the vegetative growth stage of *Bacillus*. Several genes encoding these pesticidal proteins have been isolated and characterized (see, for example, U.S. Pat. Nos. 5,366,892 and 5,840,868).

Microbial insecticides, particularly those obtained from Bacillus strains, have played an important role in agriculture as alternatives to chemical pest control. Recently, agricultural scientists have developed crop plants with enhanced 15 insect resistance by genetically engineering crop plants to produce pesticidal proteins from Bacillus. For example, corn and cotton plants have been genetically engineered to produce pesticidal proteins isolated from strains of Bt (see, e.g., Aronson (2002) Cell Mol. Life Sci. 59(3):417-425; Schnepf et al. (1998) Microbiol Mol Biol Rev. 62(3):775-806). These genetically engineered crops are now widely used in American agriculture and have provided the farmer with an environmentally friendly alternative to traditional insectcontrol methods. In addition, potatoes genetically engineered to contain pesticidal Cry toxins have been sold to the American farmer. While they have proven to be very successful commercially, these genetically engineered, insectresistant crop plants may provide resistance to only a narrow range of the economically important insect pests.

Accordingly, there remains a need for new Bt toxins with a broader range of insecticidal activity against insect pests, e.g., toxins which are active against a greater variety of insects from the order Lepidoptera. In addition, there remains a need for biopesticides having activity against a variety of insect pests and for biopesticides which have improved insecticidal activity.

## SUMMARY

Compositions and methods are provided for impacting insect pests. More specifically, the present disclosure relate to methods of impacting insects utilizing nucleotide sequences encoding insecticidal peptides to produce transformed microorganisms and plants that express an insecticidal polypeptide of the embodiments. In some aspects, the nucleotide sequences encode polypeptides that are pesticidal for at least one insect belonging to the order Lepidoptera.

In some aspects nucleic acid and fragments and variants thereof are provided, which encode polypeptides that possess pesticidal activity against insect pests (e.g. SEQ ID NO: 1 encoding SEQ ID NO: 2). The wild-type (e.g., naturally occurring) nucleotide sequence of the embodiments, which was obtained from Bt, encodes a novel insecticidal peptide. The embodiments further provide fragments and variants of the disclosed nucleotide sequence that encode biologically active (e.g., insecticidal) polypeptides.

In some aspects isolated pesticidal (e.g., insecticidal) polypeptides are provided, encoded by either a naturally occurring, or a modified (e.g., mutagenized or manipulated) nucleic acid of the embodiments. In particular aspects, pesticidal proteins of the embodiments include fragments of full-length proteins and polypeptides that are produced from mutagenized nucleic acids designed to introduce particular amino acid sequences into the polypeptides of the embodiments. In particular aspects, the polypeptides have enhanced pesticidal activity relative to the activity of the naturally occurring polypeptide from which they are derived.

In some aspects the nucleic acids of the embodiments can also be used to produce transgenic (e.g., transformed) monocot or dicot plants that are characterized by genomes that comprise at least one stably incorporated nucleotide construct comprising a coding sequence of the embodiments operably linked to a promoter that drives expression of the encoded pesticidal polypeptide. Accordingly, transformed plant cells, plant tissues, plants, and seeds thereof are also provided.

In a particular aspect, a transformed plant can be produced using a nucleic acid that has been optimized for increased expression in a host plant. For example, one of the pesticidal polypeptides of the embodiments can be back-translated to produce a nucleic acid comprising codons optimized for expression in a particular host, for example a crop plant such as a corn (*Zea mays*) plant. Expression of a coding sequence by such a transformed plant (e.g., dicot or monocot) will result in the production of a pesticidal polypeptide and confer increased insect resistance to the plant. Some embodiments provide transgenic plants expressing pesticidal polypeptides that find use in methods for impacting various insect pests.

In another aspect pesticidal or insecticidal compositions are provided containing the insecticidal polypeptides of the embodiments, and can optionally comprise further insecticidal peptides. In another aspect the application of such compositions to the environment of insect pests are provided in order to impact the insect pests.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A-1B shows an amino acid sequence alignment, using the ALIGNX® module of the Vector NTI® suite, of Cry1Ea (SEQ ID NO: 6) and amino acids 1-617 of SEQ ID NO: 2 (MP372). The sequence diversity is highlighted. The junction point between Domain I and Domain II of the MP327-Mut5 chimera (SEQ ID NO: 4) of Example 3 is indicated by a "▼".

### DETAILED DESCRIPTION

The embodiments of the disclosure are drawn to compositions and methods for impacting insect pests, particularly plant pests. More specifically, the isolated nucleic acid of the embodiments, and fragments and variants thereof, comprise 45 nucleotide sequences that encode pesticidal polypeptides (e.g., proteins). The disclosed pesticidal proteins are biologically active (e.g., pesticidal) against insect pests such as, but not limited to, insect pests of the order Lepidoptera.

The compositions of the embodiments comprise isolated 50 nucleic acids, and fragments and variants thereof, which encode pesticidal polypeptides, expression cassettes comprising nucleotide sequences of the embodiments, isolated pesticidal proteins, and pesticidal compositions. Some embodiments provide modified pesticidal polypeptides characterized by improved insecticidal activity against Lepidopterans relative to the pesticidal activity of the corresponding wild-type protein. The embodiments further provide plants and microorganisms transformed with these novel nucleic acids, and methods involving the use of such 60 nucleic acids, pesticidal compositions, transformed organisms, and products thereof in impacting insect pests.

In some embodiments an isolated nucleic acid molecule encoding an MP372 polypeptide has one or more change in the nucleic acid sequence compared to the native or genomic 65 nucleic acid sequence. In some embodiments the change in the native or genomic nucleic acid sequence includes but is

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not limited to: changes in the nucleic acid sequence due to the degeneracy of the genetic code; changes in the nucleic acid sequence due to the amino acid substitution, insertion, deletion and/or addition compared to the native or genomic sequence; removal of one or more intron; deletion of one or more upstream or downstream regulatory regions; and deletion of the 5' and/or 3' untranslated region associated with the genomic nucleic acid sequence. In some embodiments the nucleic acid molecule encoding an MP372 polypeptide is a non-genomic sequence.

The nucleic acids and nucleotide sequences of the embodiments may be used to transform any organism to produce the encoded pesticidal proteins. Methods are provided that involve the use of such transformed organisms to impact or control plant pests. The nucleic acids and nucleotide sequences of the embodiments may also be used to transform organelles such as chloroplasts (McBride et al. (1995) Biotechnology 13: 362-365; and Kota et al. (1999) *Proc. Natl. Acad. Sci. USA* 96: 1840-1845).

The embodiments further relate to the identification of fragments and variants of the naturally-occurring coding sequence that encode biologically active pesticidal proteins. The nucleotide sequences of the embodiments find direct use in methods for impacting pests, particularly insect pests such as pests of the order Lepidoptera. Accordingly, the embodiments provide new approaches for impacting insect pests that do not depend on the use of traditional, synthetic chemical insecticides. The embodiments involve the discovery of naturally-occurring, biodegradable pesticides and the genes that encode them.

The embodiments further provide fragments and variants of the naturally occurring coding sequence that also encode biologically active (e.g., pesticidal) polypeptides. The nucleic acids of the embodiments encompass nucleic acid or nucleotide sequences that have been optimized for expression by the cells of a particular organism, for example nucleic acid sequences that have been back-translated (i.e., reverse translated) using plant-preferred codons based on the amino acid sequence of a polypeptide having enhanced pesticidal activity. The embodiments further provide mutations which confer improved or altered properties on the polypeptides of the embodiments. See, e.g. U.S. Pat. No. 7,462,760.

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the embodiments.

Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. The above-defined terms are more fully defined by reference to the specification as a whole.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues (e.g., peptide nucleic acids) having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to that of naturally occurring nucleotides.

As used herein, the terms "encoding" or "encoded" when used in the context of a specified nucleic acid mean that the

nucleic acid comprises the requisite information to direct translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., 5 introns) within translated regions of the nucleic acid or may lack such intervening non-translated sequences (e.g., as in cDNA).

As used herein, "full-length sequence" in reference to a specified polynucleotide or its encoded protein means having the entire nucleic acid sequence or the entire amino acid sequence of a native (non-synthetic), endogenous sequence. A full-length polynucleotide encodes the full-length, catalytically active form of the specified protein.

As used herein, the term "antisense" used in the context of orientation of a nucleotide sequence refers to a duplex polynucleotide sequence that is operably linked to a promoter in an orientation where the antisense strand is transcribed. The antisense strand is sufficiently complementary to an endogenous transcription product such that translation of the endogenous transcription product is often inhibited. Thus, where the term "antisense" is used in the context of a particular nucleotide sequence, the term refers to the complementary strand of the reference transcription product.

The terms "polypeptide," "peptide," and "protein" are 25 used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid 30 polymers.

The terms "residue" or "amino acid residue" or "amino acid" are used interchangeably herein to refer to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "protein"). The amino acid may be a 35 naturally occurring amino acid and, unless otherwise limited, may encompass known analogues of natural amino acids that can function in a similar manner as naturally occurring amino acids.

Polypeptides of the embodiments can be produced either 40 from a nucleic acid disclosed herein, or by the use of standard molecular biology techniques. For example, a protein of the embodiments can be produced by expression of a recombinant nucleic acid of the embodiments in an appropriate host cell, or alternatively by a combination of ex 45 vivo procedures.

As used herein, the terms "isolated" and "purified" are used interchangeably to refer to nucleic acids or polypeptides or biologically active portions thereof that are substantially or essentially free from components that normally 50 accompany or interact with the nucleic acid or polypeptide as found in its naturally occurring environment. Thus, an isolated or purified nucleic acid or polypeptide is substantially free of other cellular material or culture medium when produced by recombinant techniques, or substantially free of 55 chemical precursors or other chemicals when chemically synthesized.

An "isolated" nucleic acid is generally free of sequences (such as, for example, protein-encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 60 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acids can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the 65 nucleic acids in genomic DNA of the cell from which the nucleic acid is derived.

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As used herein, the term "isolated" or "purified" as it is used to refer to a polypeptide of the embodiments means that the isolated protein is substantially free of cellular material and includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of contaminating protein. When the protein of the embodiments or biologically active portion thereof is recombinantly produced, culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Throughout the specification the word "comprising," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In some embodiments the MP372 insecticidal protein comprises an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater sequence identity to SEO ID NO: 2. In some embodiments the MP372 insecticidal protein comprises the amino acid sequence of SEQ ID NO: 2. In some embodiments the MP372 insecticidal protein of SEQ ID NO: 2 is processed from the protoxin form into a mature toxin form. In some embodiments the mature toxin comprises an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater sequence identity to amino acids 1-617 of SEQ ID NO: 2. In some embodiments the mature toxin comprises amino acids 1-617 of SEQ ID NO: 2. In some embodiments the MP372 mature toxin is fused to a heterologous Cry protein protoxin region to form a chimeric protoxin form. In some embodiments the MP372 mature toxin of amino acids 1-617 of SEQ ID NO: 2 is fused to a heterologous Cry protein protoxin region to form a chimeric protoxin form. In some embodiments the MP372 insecticidal protein comprises an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater sequence identity to SEQ ID NO: 4. In some embodiments the MP372 insecticidal protein comprises the amino acid sequence of SEQ ID NO: 4.

As used herein, the term "impacting insect pests" refers to effecting changes in insect feeding, growth, and/or behavior at any stage of development, including but not limited to: killing the insect; retarding growth; preventing reproductive capability; antifeedant activity; and the like.

As used herein, the terms "pesticidal activity" and "insecticidal activity" are used synonymously to refer to activity of an organism or a substance (such as, for example, a protein) that can be measured by, but is not limited to, pest mortality, pest weight loss, pest repellency, and other behavioral and physical changes of a pest after feeding and exposure for an appropriate length of time. Thus, an organism or substance having pesticidal activity adversely impacts at least one measurable parameter of pest fitness. For example, "pesticidal proteins" are proteins that display pesticidal activity by themselves or in combination with other proteins.

As used herein, the term "pesticidally effective amount" means a quantity of a substance or organism that has pesticidal activity when present in the environment of a pest. For each substance or organism, the pesticidally effective amount is determined empirically for each pest affected in a specific environment. Similarly, an "insecticidally effective amount" may be used to refer to a "pesticidally effective amount" when the pest is an insect pest.

As used herein, the term "recombinantly engineered" or "engineered" means the utilization of recombinant DNA

technology to introduce (e.g., engineer) a change in the protein structure based on an understanding of the protein's mechanism of action and a consideration of the amino acids being introduced, deleted, or substituted.

As used herein, the term "mutant nucleotide sequence" or "mutation" or "mutagenized nucleotide sequence" means a nucleotide sequence that has been mutagenized or altered to contain one or more nucleotide residues (e.g., base pair) that is not present in the corresponding wild-type sequence. Such mutagenesis or alteration consists of one or more additions, 10 deletions, or substitutions or replacements of nucleic acid residues. When mutations are made by adding, removing, or replacing an amino acid of a proteolytic site, such addition, removal, or replacement may be within or adjacent to the proteolytic site motif, so long as the object of the mutation 15 is accomplished (i.e., so long as proteolysis at the site is changed).

A mutant nucleotide sequence can encode a mutant insecticidal toxin showing improved or decreased insecticidal activity, or an amino acid sequence which confers improved 20 or decreased insecticidal activity on a polypeptide containing it. As used herein, the term "mutant" or "mutation" in the context of a protein a polypeptide or amino acid sequence refers to a sequence which has been mutagenized or altered to contain one or more amino acid residues that are not 25 present in the corresponding wild-type sequence. Such mutagenesis or alteration consists of one or more additions, deletions, or substitutions or replacements of amino acid residues. A mutant polypeptide shows improved or decreased insecticidal activity, or represents an amino acid 30 sequence which confers improved insecticidal activity on a polypeptide containing it. Thus, the term "mutant" or "mutation" refers to either or both of the mutant nucleotide sequence and the encoded amino acids. Mutants may be used alone or in any compatible combination with other 35 mutants of the embodiments or with other mutants. A "mutant polypeptide" may conversely show a decrease in insecticidal activity. Where more than one mutation is added to a particular nucleic acid or protein, the mutations may be added at the same time or sequentially; if sequentially, 40 mutations may be added in any suitable order.

As used herein, the term "improved insecticidal activity" or "improved pesticidal activity" refers to an insecticidal polypeptide of the embodiments that has enhanced insecticidal activity relative to the activity of its corresponding 45 wild-type protein, and/or an insecticidal polypeptide that is effective against a broader range of insects, and/or an insecticidal polypeptide having specificity for an insect that is not susceptible to the toxicity of the wild-type protein. A finding of improved or enhanced pesticidal activity requires 50 a demonstration of an increase of pesticidal activity of at least 10%, against the insect target, or at least 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 100%, 150%, 200%, or 300% or greater increase of pesticidal activity relative to the pesticidal activity of the wild-type insecticidal 55 polypeptide determined against the same insect.

For example, an improved pesticidal or insecticidal activity is provided where a wider or narrower range of insects is impacted by the polypeptide relative to the range of insects that is affected by a wild-type Bt toxin. A wider range of 60 impact may be desirable where versatility is desired, while a narrower range of impact may be desirable where, for example, beneficial insects might otherwise be impacted by use or presence of the toxin. While the embodiments are not bound by any particular mechanism of action, an improved 65 pesticidal activity may also be provided by changes in one or more characteristics of a polypeptide; for example, the

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stability or longevity of a polypeptide in an insect gut may be increased relative to the stability or longevity of a corresponding wild-type protein.

The term "toxin" as used herein refers to a polypeptide showing pesticidal activity or insecticidal activity or improved insecticidal activity. "Bt" or "Bacillus thuringiensis" toxin is intended to include the broader class of Cry toxins found in various strains of Bt, which includes such toxins as, for example, Cry1s, Cry2s, or Cry3s.

The terms "proteolytic site" or "cleavage site" refer to an amino acid sequence which confers sensitivity to a class of proteases or a particular protease such that a polypeptide containing the amino acid sequence is digested by the class of proteases or particular protease. A proteolytic site is said to be "sensitive" to the protease(s) that recognize that site. It is appreciated in the art that the efficiency of digestion will vary, and that a decrease in efficiency of digestion can lead to an increase in stability or longevity of the polypeptide in an insect gut. Thus, a proteolytic site may confer sensitivity to more than one protease or class of proteases, but the efficiency of digestion at that site by various proteases may vary. Proteolytic sites include, for example, trypsin sites, chymotrypsin sites, and elastase sites.

Research has shown that the insect gut proteases of Lepidopterans include trypsins, chymotrypsins, and elastases. See, e.g., Lenz et al. (1991) Arch. Insect Biochem. Physiol. 16: 201-212; and Hedegus et al. (2003) Arch. Insect Biochem. Physiol. 53: 30-47. For example, about 18 different trypsins have been found in the midgut of Helicoverpa armigera larvae (see Gatehouse et al. (1997) Insect Biochem. Mol. Biol. 27: 929-944). The preferred proteolytic substrate sites of these proteases have been investigated. See, e.g., Peterson et al. (1995) Insect Biochem. Mol. Biol. 25: 765-774.

Efforts have been made to understand the mechanism of action of Bt toxins and to engineer toxins with improved properties. It has been shown that insect gut proteases can affect the impact of Bt Cry proteins on the insect. Some proteases activate the Cry proteins by processing them from a "protoxin" form into a toxic form, or "toxin." See, Oppert (1999) *Arch. Insect Biochem. Phys.* 42: 1-12; and Carroll et al. (1997) *J. Invertebrate Pathology* 70: 41-49. This activation of the toxin can include the removal of the N- and C-terminal peptides from the protein and can also include internal cleavage of the protein. Other proteases can degrade the Cry proteins. See Oppert, ibid.

A comparison of the amino acid sequences of Cry toxins of different specificities reveals five highly-conserved sequence blocks. Structurally, the toxins comprise three distinct domains which are, from the N- to C-terminus: a cluster of seven alpha-helices implicated in pore formation (referred to as "domain 1"), three anti-parallel beta sheets implicated in cell binding (referred to as "domain 2"), and a beta sandwich (referred to as "domain 3"). The location and properties of these domains are known to those of skill in the art. See, for example, Li et al. (1991) Nature, 305:815-821 and Morse et al. (2001) Structure, 9:409-417. When reference is made to a particular domain, such as domain 1, it is understood that the exact endpoints of the domain with regard to a particular sequence are not critical so long as the sequence or portion thereof includes sequence that provides at least some function attributed to the particular domain. Thus, for example, when referring to "domain 1," it is intended that a particular sequence includes a cluster of seven alpha-helices, but the exact endpoints of the sequence used or referred to with regard to that cluster

are not critical. One of skill in the art is familiar with the determination of such endpoints and the evaluation of such functions

In an effort to better characterize and improve Bt toxins, strains of the bacterium Bt were studied. Crystal prepara- 5 tions prepared from cultures of the Bt strains were discovered to have pesticidal activity against numerous Lepidopteran pests (see, e.g., Experimental Example 1). An effort was undertaken to identify the nucleotide sequences encoding the crystal proteins from the selected strains, and the 10 wild-type (i.e., naturally occurring) nucleic acids of the embodiments were isolated from these bacterial strains, cloned into an expression vector, and transformed into E coli. Depending upon the characteristics of a given preparation, it was recognized that the demonstration of pesticidal 15 activity sometimes required trypsin pretreatment to activate the pesticidal proteins. Thus, it is understood that some pesticidal proteins require protease digestion (e.g., by trypsin, chymotrypsin, and the like) for activation, while other proteins are biologically active (e.g., pesticidal) in the 20 absence of activation.

Such molecules may be altered by means described, for example, U.S. Pat. No. 7,462,760. In addition, nucleic acid sequences may be engineered to encode polypeptides that contain additional mutations that confer improved or altered 25 pesticidal activity relative to the pesticidal activity of the naturally occurring polypeptide. The nucleotide sequences of such engineered nucleic acids comprise mutations not found in the wild type sequences.

The mutant polypeptides of the embodiments are generally prepared by a process that involves the steps of: obtaining a nucleic acid sequence encoding a Cry family polypeptide; analyzing the structure of the polypeptide to identify particular "target" sites for mutagenesis of the underlying gene sequence based on a consideration of the 35 proposed function of the target domain in the mode of action of the toxin; introducing one or more mutations into the nucleic acid sequence to produce a desired change in one or more amino acid residues of the encoded polypeptide sequence; and assaying the polypeptide produced for pesticidal activity.

Many of the Bt insecticidal toxins are related to various degrees by similarities in their amino acid sequences and tertiary structure and means for obtaining the crystal structures of Bt toxins are well known. Exemplary high-resolu- 45 tion crystal structure solution of both the Cry3A and Cry3B polypeptides are available in the literature. The solved structure of the Cry3A gene (Li et al. (1991) Nature 353: 815-821) provides insight into the relationship between structure and function of the toxin. A combined consider- 50 ation of the published structural analyses of Bt toxins and the reported function associated with particular structures, motifs, and the like indicates that specific regions of the toxin are correlated with particular functions and discrete steps of the mode of action of the protein. For example, 55 many toxins isolated from Bt are generally described as comprising three domains: a seven-helix bundle that is involved in pore formation, a three-sheet domain that has been implicated in receptor binding, and a beta-sandwich motif (Li et al. (1991) Nature 305: 815-821).

As reported in U.S. Pat. Nos. 7,105,332, and 7,462,760, the toxicity of Cry proteins can be improved by targeting the region located between alpha helices 3 and 4 of domain 1 of the toxin. This theory was premised on a body of knowledge concerning insecticidal toxins, including: 1) that alpha helices 4 and 5 of domain 1 of Cry3A toxins had been reported to insert into the lipid bilayer of cells lining the midgut of

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susceptible insects (Gazit et al. (1998) *Proc. Natl. Acad. Sci. USA* 95: 12289-12294); 2) the inventors' knowledge of the location of trypsin and chymotrypsin cleavage sites within the amino acid sequence of the wild-type protein; 3) the observation that the wild-type protein was more active against certain insects following in vitro activation by trypsin or chymotrypsin treatment; and 4) reports that digestion of toxins from the 3' end resulted in decreased toxicity to insects.

A series of mutations may be created and placed in a variety of background sequences to create novel polypeptides having enhanced or altered pesticidal activity. See, e.g., U.S. Pat. No. 7,462,760. These mutants include, but are not limited to: the addition of at least one more proteasesensitive site (e.g., trypsin cleavage site) in the region located between helices 3 and 4 of domain 1; the replacement of an original protease-sensitive site in the wild-type sequence with a different protease-sensitive site; the addition of multiple protease-sensitive sites in a particular location; the addition of amino acid residues near protease-sensitive site(s) to alter folding of the polypeptide and thus enhance digestion of the polypeptide at the protease-sensitive site(s); and adding mutations to protect the polypeptide from degradative digestion that reduces toxicity (e.g., making a series of mutations wherein the wild-type amino acid is replaced by valine to protect the polypeptide from digestion). Mutations may be used singly or in any combination to provide polypeptides of the embodiments.

In this manner, the embodiments provide sequences comprising a variety of mutations, such as, for example, a mutation that comprises an additional, or an alternative, protease-sensitive site located between alpha-helices 3 and 4 of domain 1 of the encoded polypeptide.

A mutation which is an additional or alternative protease-sensitive site may be sensitive to several classes of proteases such as serine proteases, which include trypsin and chymotrypsin, or enzymes such as elastase. Thus, a mutation which is an additional or alternative protease-sensitive site may be designed so that the site is readily recognized and/or cleaved by a category of proteases, such as mammalian proteases or insect proteases. A protease-sensitive site may also be designed to be cleaved by a particular class of enzymes or a particular enzyme known to be produced in an organism, such as, for example, a chymotrypsin produced by the corn earworm *Heliothis zea* (Lenz et al. (1991) *Arch. Insect Biochem. Physiol.* 16: 201-212). Mutations may also confer resistance to proteolytic digestion, for example, to digestion by chymotrypsin at the C-terminus of the peptide.

The presence of an additional and/or alternative proteasesensitive site in the amino acid sequence of the encoded polypeptide can improve the pesticidal activity and/or specificity of the polypeptide encoded by the nucleic acids of the embodiments. Accordingly, the nucleotide sequences of the embodiments can be recombinantly engineered or manipulated to produce polypeptides having improved or altered insecticidal activity and/or specificity compared to that of an unmodified wild-type toxin. In addition, the mutations disclosed herein may be placed in or used in conjunction with other nucleotide sequences to provide improved properties. For example, a protease-sensitive site that is readily cleaved by insect chymotrypsin, e.g., a chymotrypsin found in the bertha armyworm or the corn earworm (Hegedus et al. (2003) Arch. Insect Biochem. Physiol. 53: 30-47; and Lenz et al. (1991) Arch. Insect Biochem. Physiol. 16: 201-212), may be placed in a Cry background sequence to provide

improved toxicity to that sequence. In this manner, the embodiments provide toxic polypeptides with improved properties.

For example, a mutagenized Cry nucleotide sequence can comprise additional mutants that comprise additional 5 codons that introduce a second trypsin-sensitive amino acid sequence (in addition to the naturally occurring trypsin site) into the encoded polypeptide. An alternative addition mutant of the embodiments comprises additional codons designed to introduce at least one additional different protease-sensi- 10 tive site into the polypeptide; for example, a chymotrypsinsensitive site located immediately 5' or 3' of the naturally occurring trypsin site. Alternatively, substitution mutants may be created in which at least one codon of the nucleic acid that encodes the naturally occurring protease-sensitive 15 site is destroyed and alternative codons are introduced into the nucleic acid sequence in order to provide a different (e.g., substitute) protease-sensitive site. A replacement mutant may also be added to a Cry sequence in which the naturallyoccurring trypsin cleavage site present in the encoded poly- 20 peptide is destroyed and a chymotrypsin or elastase cleavage site is introduced in its place.

It is recognized that any nucleotide sequence encoding the amino acid sequences that are proteolytic sites or putative proteolytic sites (for example, sequences such as RR, or 25 LKM) can be used and that the exact identity of the codons used to introduce any of these cleavage sites into a variant polypeptide may vary depending on the use, i.e., expression in a particular plant species. It is also recognized that any of the disclosed mutations can be introduced into any poly- 30 nucleotide sequence of the embodiments that comprises the codons for amino acid residues that provide the native trypsin cleavage site that is targeted for modification. Accordingly, variants of either full-length toxins or fragments thereof can be modified to contain additional or 35 alternative cleavage sites, and these embodiments are intended to be encompassed by the scope of the embodiments disclosed herein.

It will be appreciated by those of skill in the art that any useful mutation may be added to the sequences of the 40 embodiments so long as the encoded polypeptides retain pesticidal activity. Thus, sequences may also be mutated so that the encoded polypeptides are resistant to proteolytic digestion by chymotrypsin. More than one recognition site can be added in a particular location in any combination, and 45 multiple recognition sites can be added to or removed from the toxin. Thus, additional mutations can comprise three, four, or more recognition sites. It is to be recognized that multiple mutations can be engineered in any suitable polynucleotide sequence; accordingly, either full-length 50 sequences or fragments thereof can be modified to contain additional or alternative cleavage sites as well as to be resistant to proteolytic digestion. In this manner, the embodiments provide Cry toxins containing mutations that improve pesticidal activity as well as improved compositions and 55 methods for impacting pests using other Bt toxins.

Mutations may protect the polypeptide from protease degradation, for example by removing putative proteolytic sites such as putative serine protease sites and elastase recognition sites from different areas. Some or all of such 60 putative sites may be removed or altered so that proteolysis at the location of the original site is decreased. Changes in proteolysis may be assessed by comparing a mutant polypeptide with wild-type toxins or by comparing mutant toxins which differ in their amino acid sequence. Putative proteolytic sites and proteolytic sites include, but are not limited to, the following sequences: RR, a trypsin cleavage site;

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LKM, a chymotrypsin site; and a trypsin site. These sites may be altered by the addition or deletion of any number and kind of amino acid residues, so long as the pesticidal activity of the polypeptide is increased. Thus, polypeptides encoded by nucleotide sequences comprising mutations will comprise at least one amino acid change or addition relative to the native or background sequence, or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 35, 38, 40, 45, 47, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, or 280 or more amino acid changes or additions. Pesticidal activity of a polypeptide may also be improved by truncation of the native or full-length sequence, as is known in the art.

In another embodiment fusion proteins are provided that include within its amino acid sequence an amino acid sequence comprising an MP372 polypeptide or chimeric MP372 polypeptide of the disclosure. Methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art. Polynucleotides encoding an MP372 polypeptide may be fused to signal sequences which will direct the localization of the MP372 polypeptide to particular compartments of a prokaryotic or eukaryotic cell and/or direct the secretion of the MP372 polypeptide of the embodiments from a prokaryotic or eukaryotic cell. For example, in E. coli, one may wish to direct the expression of the protein to the periplasmic space. Examples of signal sequences or proteins (or fragments thereof) to which the MP372 polypeptide may be fused in order to direct the expression of the polypeptide to the periplasmic space of bacteria include, but are not limited to, the pelB signal sequence, the maltose binding protein (MBP) signal sequence, MBP, the ompA signal sequence, the signal sequence of the periplasmic E. coli heat-labile enterotoxin B-subunit and the signal sequence of alkaline phosphatase. Several vectors are commercially available for the construction of fusion proteins which will direct the localization of a protein, such as the pMAL series of vectors (particularly the pMAL-p series) available from New England Biolabs. In a specific embodiment, the MP372 polypeptide may be fused to the pelB pectate lyase signal sequence to increase the efficiency of expression and purification of such polypeptides in Gram-negative bacteria (see, U.S. Pat. Nos. 5,576,195 and 5,846,818). Plant plastid transit peptide/polypeptide fusions are well known in the art. Apoplast transit peptides such as rice or barley alphaamylase secretion signal are also well known in the art. The plastid transit peptide is generally fused N-terminal to the polypeptide to be targeted (e.g., the fusion partner). In one embodiment, the fusion protein consists essentially of the plastid transit peptide and the MP372 polypeptide to be targeted. In another embodiment, the fusion protein comprises the plastid transit peptide and the polypeptide to be targeted. In such embodiments, the plastid transit peptide is preferably at the N-terminus of the fusion protein. However, additional amino acid residues may be N-terminal to the plastid transit peptide providing that the fusion protein is at least partially targeted to a plastid. In a specific embodiment, the plastid transit peptide is in the N-terminal half, N-terminal third or N-terminal quarter of the fusion protein. Most or all of the plastid transit peptide is generally cleaved from the fusion protein upon insertion into the plastid. The position of cleavage may vary slightly between plant species, at different plant developmental stages, as a result of specific intercellular conditions or the particular combination of transit peptide/fusion partner used. In one embodiment, the plastid transit peptide cleavage is homogenous

such that the cleavage site is identical in a population of fusion proteins. In another embodiment, the plastid transit peptide is not homogenous, such that the cleavage site varies by 1-10 amino acids in a population of fusion proteins. The plastid transit peptide can be recombinantly fused to a 5 second protein in one of several ways. For example, a restriction endonuclease recognition site can be introduced into the nucleotide sequence of the transit peptide at a position corresponding to its C-terminal end and the same or a compatible site can be engineered into the nucleotide 10 sequence of the protein to be targeted at its N-terminal end. Care must be taken in designing these sites to ensure that the coding sequences of the transit peptide and the second protein are kept "in frame" to allow the synthesis of the desired fusion protein. In some cases, it may be preferable 15 to remove the initiator methionine of the second protein when the new restriction site is introduced. The introduction of restriction endonuclease recognition sites on both parent molecules and their subsequent joining through recombinant DNA techniques may result in the addition of one or more 20 extra amino acids between the transit peptide and the second protein. This generally does not affect targeting activity as long as the transit peptide cleavage site remains accessible and the function of the second protein is not altered by the addition of these extra amino acids at its N-terminus. Alter- 25 natively, one skilled in the art can create a precise cleavage site between the transit peptide and the second protein (with or without its initiator methionine) using gene synthesis (Stemmer, et al., (1995) Gene 164:49-53) or similar methods. In addition, the transit peptide fusion can intentionally 30 include amino acids downstream of the cleavage site. The amino acids at the N-terminus of the mature protein can affect the ability of the transit peptide to target proteins to plastids and/or the efficiency of cleavage following protein import. This may be dependent on the protein to be targeted. 35 See, e.g., Comai, et al., (1988) J. Biol. Chem. 263(29): 15104-9. In some embodiments the MP372 polypeptide is fused to a heterologous signal peptide or heterologous transit peptide.

In some embodiments the insecticidal protein is a chime-40 ric Cry1E polypeptide comprising: a) Domain I of a first Cry1E protein and b) Domain II and Domain III of a second Cry1E protein of SEQ ID NO: 2 or a fragment or variant thereof. In some embodiments the insecticidal protein is a chimeric Cry1E polypeptide comprising: a) Domain I of the 45 Cry1Ea protein (Accession No. X53985) of SEQ ID NO: 6, and b) Domain II and Domain III of the Cry1 protein of SEO ID NO: 2. In some embodiments the insecticidal protein is a chimeric Cry1E polypeptide comprising: a) a Domain I having at least 95% sequence identity to amino acids 36-253 50 of SEQ ID NO: 6, and b) Domain II and Domain III having at least 95% sequence identity to amino acids 259-617 of SEQ ID NO: 2. In some embodiments the insecticidal protein is a chimeric Cry1E polypeptide comprising amino acids 36-259 of SEQ ID NO: 6, and amino acids 259-617 of 55 SEQ ID NO: 2. In some embodiments the insecticidal protein is a chimeric Cry1E polypeptide comprising amino acids 1-259 of SEQ ID NO: 6, and amino acids 259-617 of SEQ ID NO: 2. In some embodiments the chimeric Cry1E polypeptide has at least 80%, 85%, 90%, 91%, 92%, 93%, 60 94%, 95%, 96%, 97%, 98%, 99% or greater sequence identity to SEQ ID NO: 4. In some embodiments the chimeric Cry1E polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

Compositions of the embodiments include nucleic acids, 65 and fragments and variants thereof that encode pesticidal polypeptides. In particular, the embodiments provide for

isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequence shown in SEQ ID NO: 2 or SEQ ID NO: 4, or the nucleotide sequences encoding said amino acid sequence, for example the nucleotide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 3, and fragments and variants thereof.

Also of interest are optimized nucleotide sequences encoding the pesticidal proteins of the embodiments. As used herein, the phrase "optimized nucleotide sequences" refers to nucleic acids that are optimized for expression in a particular organism, for example a plant. Optimized nucleotide sequences may be prepared for any organism of interest using methods known in the art. See, for example, U.S. Pat. No. 7,462,760, which describes an optimized nucleotide sequence encoding a disclosed pesticidal protein. This procedure is described in more detail by Murray et al. (1989) *Nucleic Acids Res.* 17:477-498. Optimized nucleotide sequences find use in increasing expression of a pesticidal protein in a plant, for example monocot plants of the Gramineae (Poaceae) family such as, for example, a maize or corn plant.

The embodiments further provide isolated pesticidal (e.g., insecticidal) polypeptides encoded by either a naturally-occurring or modified nucleic acid of the embodiments. More specifically, the embodiments provide polypeptides comprising an amino acid sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 4, and the polypeptides encoded by nucleic acids described herein, for example those set forth in SEQ ID NO: 1 or SEQ ID NO: 3, and fragments and variants thereof.

In particular embodiments, pesticidal proteins of the embodiments provide full-length insecticidal polypeptides, fragments of full-length insecticidal polypeptides, and variant polypeptides that are produced from mutagenized nucleic acids designed to introduce particular amino acid sequences into polypeptides of the embodiments. In particular embodiments, the amino acid sequences that are introduced into the polypeptides comprise a sequence that provides a cleavage site for an enzyme such as a protease.

It is known in the art that the pesticidal activity of Bt toxins is typically activated by cleavage of the peptide in the insect gut by various proteases. Because peptides may not always be cleaved with complete efficiency in the insect gut, fragments of a full-length toxin may have enhanced pesticidal activity in comparison to the full-length toxin itself. Thus, some of the polypeptides of the embodiments include fragments of a full-length insecticidal polypeptide, and some of the polypeptide fragments, variants, and mutations will have enhanced pesticidal activity relative to the activity of the naturally occurring insecticidal polypeptide from which they are derived, particularly if the naturally occurring insecticidal polypeptide is not activated in vitro with a protease prior to screening for activity. Thus, the present application encompasses truncated versions or fragments of the sequences.

Mutations may be placed into any background sequence, including such truncated polypeptides, so long as the polypeptide retains pesticidal activity. One of skill in the art can readily compare two or more proteins with regard to pesticidal activity using assays known in the art or described elsewhere herein. It is to be understood that the polypeptides of the embodiments can be produced either by expression of a nucleic acid disclosed herein, or by the use of standard molecular biology techniques.

It is recognized that the pesticidal proteins may be oligomeric and will vary in molecular weight, number of residues, component peptides, activity against particular pests,

and other characteristics. However, by the methods set forth herein, proteins active against a variety of pests may be isolated and characterized. The pesticidal proteins of the embodiments can be used in combination with other Bt toxins or other insecticidal proteins to increase insect target 5 range. Furthermore, the use of the pesticidal proteins of the embodiments in combination with other Bt toxins or other insecticidal principles of a distinct nature has particular utility for the prevention and/or management of insect resistance. Other insecticidal agents include protease inhibitors (both serine and cysteine types),  $\alpha$ -amylase, and peroxidase.

Fragments and variants of the nucleotide and amino acid sequences and the polypeptides encoded thereby are also encompassed by the embodiments. As used herein the term 15 "fragment" refers to a portion of a nucleotide sequence of a polynucleotide or a portion of an amino acid sequence of a polypeptide of the embodiments. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native or corresponding full-length 20 protein and hence possess pesticidal activity. Thus, it is acknowledged that some of the polynucleotide and amino acid sequences of the embodiments can correctly be referred to as both fragments and mutants.

It is to be understood that the term "fragment," as it is 25 used to refer to nucleic acid sequences of the embodiments, also encompasses sequences that are useful as hybridization probes. This class of nucleotide sequences generally does not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at 30 least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the proteins of the embodiments.

A fragment of a nucleotide sequence of the embodiments that encodes a biologically active portion of a pesticidal 35 protein of the embodiments will encode at least 15, 25, 30, 50, 100, 200, 250 or 300 contiguous amino acids, or up to the total number of amino acids present in a pesticidal polypeptide of the embodiments (for example, 1176 amino acids for SEQ ID NO: 2). Thus, it is understood that the 40 embodiments also encompass polypeptides that are fragments of the exemplary pesticidal proteins of the embodiments and having lengths of at least 15, 25, 30, 50, 100, 200, 250 or 300 contiguous amino acids, or up to the total number of amino acids present in a pesticidal polypeptide of the 45 embodiments (for example, 1176 amino acids for SEQ ID NO: 2). Fragments of a nucleotide sequence of the embodiments that are useful as hybridization probes or PCR primers generally need not encode a biologically active portion of a pesticidal protein. Thus, a fragment of a nucleic acid of the 50 embodiments may encode a biologically active portion of a pesticidal protein, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed herein. A biologically active portion of a pesticidal protein can be prepared by isolating a portion of one of the 55 nucleotide sequences of the embodiments, expressing the encoded portion of the pesticidal protein (e.g., by recombinant expression in vitro), and assessing the activity of the encoded portion of the pesticidal protein.

Nucleic acids that are fragments of a nucleotide sequence 60 of the embodiments comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 850, 900 or 950 nucleotides, or up to the number of nucleotides present in a nucleotide sequence disclosed herein (for example, 3528 nucleotides for SEQ ID NO: 1). Particular 65 embodiments envision fragments derived from (e.g., produced from) a first nucleic acid of the embodiments, wherein

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the fragment encodes a truncated toxin characterized by pesticidal activity. Truncated polypeptides encoded by the polynucleotide fragments of the embodiments are characterized by pesticidal activity that is either equivalent to, or improved, relative to the activity of the corresponding full-length polypeptide encoded by the first nucleic acid from which the fragment is derived. It is envisioned that such nucleic acid fragments of the embodiments may be truncated at the 3' end of the native or corresponding full-length coding sequence. Nucleic acid fragments may also be truncated at both the 5' and 3' end of the native or corresponding full-length coding sequence.

The term "variants" is used herein to refer to substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the pesticidal polypeptides of the embodiments. Those having ordinary skill in the art will readily appreciate that due to the degeneracy of the genetic code, a multitude of nucleotide sequences encoding of the present disclosure exist.

Where appropriate, a nucleic acid may be optimized for increased expression in the host organism. Thus, where the host organism is a plant, the synthetic nucleic acids can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri, (1990) Plant Physiol. 92:1-11 for a discussion of host-preferred codon usage. For example, although nucleic acid sequences of the embodiments may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray et al. (1989) Nucleic Acids Res. 17:477-498). Thus, the maizepreferred codon for a particular amino acid may be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants is listed in Table 4 of Murray, et al., supra. Methods are available in the art for synthesizing plant-preferred genes. See, for example, Murray, et al., (1989) Nucleic Acids Res. 17:477-498, and Liu H et al. Mol Bio Rep 37:677-684, 2010, herein incorporated by reference. A Zea maize codon usage table can be also found at kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4577, which can be accessed using the www prefix. A maize optimal codon analysis (adapted from Liu H et al. Mol Bio Rep 37:677-684, 2010).

A  $Glycine\ max$  codon usage table can be found at kazusa.or.jp/codon/cgi-bin/

showcodon.cgi?species=3847&aa=1&style=N, which can be accessed using the www prefix.

The skilled artisan will further appreciate that changes can be introduced by mutation of the nucleic acid sequences thereby leading to changes in the amino acid sequence of the encoded polypeptides, without altering the biological activity of the proteins. Thus, variant nucleic acid molecules can be created by introducing one or more nucleotide substitutions, additions and/or deletions into the corresponding nucleic acid sequence disclosed herein, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Such variant nucleic acid sequences are also encompassed by the present disclosure.

Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology

techniques, such as, for example, polymerase chain reaction (PCR) and hybridization techniques as outlined herein.

Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a pesticidal protein of the embodiments, such as a mutant toxin. Generally, variants of a particular nucleotide sequence of the embodiments will have at least about 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters. A variant of a nucleotide sequence of the embodiments may differ from that sequence by as few as 1-15 nucleotides, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 nucleotide.

Variants of a particular nucleotide sequence of the embodiments (i.e., an exemplary nucleotide sequence) can also be evaluated by comparison of the percent sequence 20 identity between the polypeptide encoded by a variant nucleotide sequence and the polypeptide encoded by the reference nucleotide sequence. Thus, for example, isolated nucleic acids that encode a polypeptide with a given percent sequence identity to the polypeptide of SEQ ID NO: 2 or 25 SEQ ID NO: 4 are disclosed. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs described elsewhere herein using default parameters. Where any given pair of polynucleotides of the embodiments is evaluated by comparison 30 of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 35 99% or more sequence identity.

As used herein, the term "variant protein" encompasses polypeptides that are derived from a native protein by: deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the 40 native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Accordingly, the term "variant protein" encompasses biologically active fragments of a native protein that comprise a sufficient number of contiguous amino acid residues to retain the biological activity of the native protein, i.e., to have pesticidal activity. Such pesticidal activity may be different or improved relative to the native protein or it may be unchanged, so long as pesticidal activity 50 is retained.

Variant proteins encompassed by the embodiments are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, pesticidal activity as described herein. Such variants may 55 result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native pesticidal protein of the embodiments will have at least about 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 60 99%, or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the embodiments may differ from that protein by as few as 1-15 65 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

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In some embodiments the polypeptide has a modified physical property. As used herein, the term "physical property" refers to any parameter suitable for describing the physical-chemical characteristics of a protein. As used herein, "physical property of interest" and "property of interest" are used interchangeably to refer to physical properties of proteins that are being investigated and/or modified. Examples of physical properties include, but are not limited to net surface charge and charge distribution on the protein surface, net hydrophobicity and hydrophobic residue distribution on the protein surface, surface charge density, surface hydrophobicity density, total count of surface ionizable groups, surface tension, protein size and its distribution in solution, melting temperature, heat capacity, and second virial coefficient. Examples of physical properties also include, but are not limited to solubility, folding, stability, and digestibility. In some embodiments the polypeptide has increased digestibility of proteolytic fragments in an insect gut. Models for digestion by simulated simulated gastric fluids are known to one skilled in the art (Fuchs, R. L. and J. D. Astwood. Food Technology 50: 83-88, 1996; Astwood, J. D., et al Nature Biotechnology 14: 1269-1273, 1996; Fu T J et al J. Agric Food Chem. 50: 7154-7160, 2002).

The embodiments further encompass a microorganism that is transformed with at least one nucleic acid of the embodiments, with an expression cassette comprising the nucleic acid, or with a vector comprising the expression cassette. In some embodiments, the microorganism is one that multiplies on plants. An embodiment of the disclosure relates to an encapsulated pesticidal protein which comprises a transformed microorganism capable of expressing at least one pesticidal protein of the embodiments.

The embodiments provide pesticidal compositions comprising a transformed microorganism of the embodiments. In such embodiments, the transformed microorganism is generally present in the pesticidal composition in a pesticidally effective amount, together with a suitable carrier. The embodiments also encompass pesticidal compositions comprising an isolated protein of the embodiments, alone or in combination with a transformed organism of the embodiments and/or an encapsulated pesticidal protein of the embodiments, in an insecticidally effective amount, together with a suitable carrier.

The embodiments further provide a method of increasing insect target range by using a pesticidal protein of the embodiments in combination with at least one other or "second" pesticidal protein. Any pesticidal protein known in the art can be employed in the methods of the embodiments. Such pesticidal proteins include, but are not limited to, Bt toxins, protease inhibitors,  $\alpha$ -amylases, and peroxidases.

The embodiments also encompass transformed or transgenic plants comprising at least one nucleotide sequence of the embodiments. In some embodiments, the plant is stably transformed with a nucleotide construct comprising at least one nucleotide sequence of the embodiments operably linked to a promoter that drives expression in a plant cell. As used herein, the terms "transformed plant" and "transgenic plant" refer to a plant that comprises within its genome a heterologous polynucleotide. Generally, the heterologous polynucleotide is stably integrated within the genome of a transgenic or transformed plant such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette.

It is to be understood that as used herein the term "transgenic" includes any cell, cell line, callus, tissue, plant part, or plant the genotype of which has been altered by the

presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

As used herein, the term "plant" includes whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, and progeny of same. Parts of transgenic plants are within the scope of the embodiments and comprise, for example, plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, 20 roots, root tips, anthers, and the like, originating in transgenic plants or their progeny previously transformed with a DNA molecule of the embodiments and therefore consisting at least in part of transgenic cells. The class of plants that can be used in the methods of the embodiments is generally as 25 broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

While the embodiments do not depend on a particular biological mechanism for increasing the resistance of a plant to a plant pest, expression of the nucleotide sequences of the embodiments in a plant can result in the production of the pesticidal proteins of the embodiments and in an increase in the resistance of the plant to a plant pest. The plants of the embodiments find use in agriculture in methods for impacting insect pests. Certain embodiments provide transformed crop plants, such as, for example, maize plants, which find use in methods for impacting insect pests of the plant, such as, for example, Lepidopteran pests.

A "subject plant or plant cell" is one in which genetic alteration, such as transformation, has been affected as to a gene of interest, or is a plant or plant cell which is descended from a plant or cell so altered and which comprises the alteration. A "control" or "control plant" or "control plant 45 cell" provides a reference point for measuring changes in phenotype of the subject plant or plant cell.

A control plant or plant cell may comprise, for example:
(a) a wild-type plant or cell, i.e., of the same genotype as the starting material for the genetic alteration which resulted in the subject plant or cell; (b) a plant or plant cell of the same genotype as the starting material but which has been transformed with a null construct (i.e., with a construct which has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a plant or plant cell which is a non-transformed segregant among progeny of a subject plant or plant cell; (d) a plant or plant cell genetically identical to the subject plant or plant cell but which is not exposed to conditions or stimuli that would induce expression of the gene of interest; or (e) the subject plant or plant cell itself, under conditions in which the gene of interest is not expressed.

One of skill in the art will readily acknowledge that advances in the field of molecular biology such as site-specific and random mutagenesis, polymerase chain reaction 65 methodologies, and protein engineering techniques provide an extensive collection of tools and protocols suitable for

use to alter or engineer both the amino acid sequence and underlying genetic sequences of proteins of agricultural interest

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Thus, the proteins of the embodiments may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the pesticidal proteins can be prepared by introducing mutations into a synthetic nucleic acid (e.g., DNA molecule). Methods for mutagenesis and nucleic acid alterations are well known in the art. For example, designed changes can be introduced using an oligonucleotide-mediated site-directed mutagenesis technique. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel et al. (1987) *Methods in Enzymol.* 154:367-382; U.S. Pat. No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York), and the references cited therein.

The mutagenized nucleotide sequences of the embodiments may be modified so as to change about 1, 2, 3, 4, 5, 6, 8, 10, 12 or more of the amino acids present in the primary sequence of the encoded polypeptide. Alternatively, even more changes from the native sequence may be introduced such that the encoded protein may have at least about 1% or 2%, or about 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, or even about 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20%, 21%, 22%, 23%, 24%, or 25%, 30%, 35%, or 40% or more of the codons altered, or otherwise modified compared to the corresponding wild-type protein. In the same manner, the encoded protein may have at least about 1% or 2%, or about 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, or even about 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20%, 21%, 22%, 23%, 24%, or 25%, 30%, 35%, or 40% or more additional codons compared to the corresponding wild-type protein. It should be understood that the mutagenized nucleotide sequences of the embodiments are intended to encompass biologically functional, equivalent peptides which have pesticidal activity, such as an improved pesticidal activity as determined by antifeedant properties against European corn borer larvae. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded.

One of skill in the art would recognize that amino acid additions and/or substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, charge, size, and the like. Exemplary amino acid substitution groups that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine, and isoleucine.

Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff et al. (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be made.

Thus, the genes and nucleotide sequences of the embodiments include both the naturally occurring sequences and mutant forms. Likewise, the proteins of the embodiments encompass both naturally occurring proteins and variations (e.g., truncated polypeptides) and modified (e.g., mutant) forms thereof. Such variants will continue to possess the desired pesticidal activity. Obviously, the mutations that will be made in the nucleotide sequence encoding the variant

must not place the sequence out of reading frame and generally will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the protein 5 sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be 10 evaluated by routine screening assays, such as insect-feeding assays. See, for example, Marrone et al. (1985) *J. Econ. Entomol.* 78: 290-293 and Czapla and Lang (1990) *J. Econ. Entomol.* 83: 2480-2485, herein incorporated by reference.

Variant nucleotide sequences and proteins also encompass 15 sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different coding sequences can be manipulated to create a new pesticidal protein possessing the desired properties. In this manner, libraries of 20 recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined in vitro or in vivo. For example, using this approach, full-length coding sequences, 25 sequence motifs encoding a domain of interest, or any fragment of a nucleotide sequence of the embodiments may be shuffled between the nucleotide sequences of the embodiments and corresponding portions of other known Cry nucleotide sequences to obtain a new gene coding for a 30 protein with an improved property of interest.

Properties of interest include, but are not limited to, pesticidal activity per unit of pesticidal protein, protein stability, and toxicity to non-target species particularly humans, livestock, and plants and microbes that express the 35 pesticidal polypeptides of the embodiments. The embodiments are not bound by a particular shuffling strategy, only that at least one nucleotide sequence of the embodiments, or part thereof, is involved in such a shuffling strategy. Shuffling may involve only nucleotide sequences disclosed 40 herein or may additionally involve shuffling of other nucleotide sequences known in the art. Strategies for DNA shuffling are known in the art. See, for example, Stemmer (1994) Proc. Natl. Acad. Sci. USA 91:10747-10751; Stemmer (1994) Nature 370:389-391; Crameri et al. (1997) Nature 45 Biotech. 15:436-438; Moore et al. (1997) J. Mol. Biol. 272:336-347; Zhang et al. (1997) Proc. Natl. Acad. Sci. USA 94:4504-4509; Crameri et al. (1998) Nature 391:288-291; and U.S. Pat. Nos. 5,605,793 and 5,837,458.

The nucleotide sequences of the embodiments can also be 50 used to isolate corresponding sequences from other organisms, particularly other bacteria, and more particularly other Bacillus strains. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the 55 sequences set forth herein. Sequences that are selected based on their sequence identity to the entire sequences set forth herein or to fragments thereof are encompassed by the embodiments. Such sequences include sequences that are orthologs of the disclosed sequences. The term "orthologs" refers to genes derived from a common ancestral gene and which are found in different species as a result of speciation. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share substantial identity as defined else- 65 where herein. Functions of orthologs are often highly conserved among species.

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In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any organism of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual (2d ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.), hereinafter "Sambrook". See also Innis et al., eds. (1990) PCR Protocols: A Guide to Methods and Applications (Academic Press, New York); Innis and Gelfand, eds. (1995) PCR Strategies (Academic Press, New York); and Innis and Gelfand, eds. (1999) PCR Methods Manual (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as <sup>32</sup>P or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the sequences of the embodiments. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook.

For example, an entire sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique to the sequences of the embodiments and are generally at least about 10 or 20 nucleotides in length. Such probes may be used to amplify corresponding Cry sequences from a chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in an organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook).

Hybridization of such sequences may be carried out under stringent conditions. The term "stringent conditions" or "stringent hybridization conditions" as used herein refers to conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold, 5-fold, or 10-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 or 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for long probes (e.g., greater than 50 nucleotides).

Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulfate) at 37° C., and a wash in 1× to 52×SSC (20×SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55° C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37° C., and a wash in 0.5× to 1×SSC at 55 to 60° C. Exemplary high stringency conditions include hybridization 10 in 50% formamide, 1 M NaCl, 1% SDS at 37° C., and a final wash in 0.1×SSC at 60 to 65° C. for at least about 20 minutes. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. The duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the  $T_m$  (thermal melting point) can be approximated from the equation of Meinkoth and Wahl (1984) Anal. 20 Biochem. 138:267-284: T<sub>m</sub>=81.5° C.+16.6 (log M)+0.41 (% GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, "% form" is the percentage of formamide in the hybridization solution, and 25 L is the length of the hybrid in base pairs. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. Washes are typically performed at least until equilibrium is reached and a low background level 30 of hybridization is achieved, such as for 2 hours, 1 hour, or

 $T_m$  is reduced by about 1° C. for each 1% of mismatching; thus,  $T_m$ , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. 35 For example, if sequences with  $\geq 90\%$  identity are sought, the  $T_m$  can be decreased 10° C. Generally, stringent conditions are selected to be about 5° C. lower than the  $T_m$  for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4° C. lower than the  $T_m$ ; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10° C. lower than the  $T_m$ ; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20° C. lower than the  $T_m$ .

Using the equation, hybridization and wash compositions, and desired  $T_m$ , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of 50 mismatching results in a  $T_m$  of less than 45° C. (aqueous solution) or 32° C. (formamide solution), the SSC concentration can be increased so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in 55 Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes, Part I, Chapter 2 (Elsevier, New York); and Ausubel et al., eds. (1995) Current Protocols in Molecular Biology, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See also Sambrook. Thus, isolated 60 sequences that encode a Cry protein of the embodiments and hybridize under stringent conditions to the Cry sequences disclosed herein, or to fragments thereof, are encompassed by the embodiments.

The following terms are used to describe the sequence 65 relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison win-

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dow", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local alignment algorithm of Smith et al. (1981) *Adv. Appl. Math.* 2:482; the global alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-local alignment method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 872264, as modified in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys Inc., 9685 Scranton Road, San Diego, Calif., USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins et al. (1988) Gene 73:237-244 (1988); Higgins et al. (1989) CABIOS 5:151-153; Corpet et al. (1988) Nucleic Acids Res. 16:10881-90; Huang et al. (1992) CABIOS 8:155-65; and Pearson et al. (1994) Meth. Mol. Biol. 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) supra. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul et al (1990) J. Mol. Biol. 215:403 are based on the algorithm of Karlin and Altschul (1990) supra. BLAST nucleotide searches can be performed with the BLASTN program, score=100, wordlength=12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the embodiments. BLAST protein searches can be performed with the BLASTX program, score=50, wordlength=3, to obtain amino acid sequences homologous to a protein or polypeptide of the embodiments. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant

relationships between molecules. See Altschul et al. (1997) supra. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See the National Center for Biotechnology Information website on the world wide web at ncbi.hlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP 10 Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, 15 and the BLOSUM62 scoring matrix; or any equivalent program thereof. The term "equivalent program" as used herein refers to any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches 20 and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

GAP uses the algorithm of Needleman and Wunsch (1970) supra, to find the alignment of two complete sequences that maximizes the number of matches and mini- 25 mizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must 30 make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap exten- 35 sion penalty values in Version 10 of the GCG Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties 40 can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the GCG Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of 65 sequence identity is used in reference to proteins it is recognized that residue positions which are not identical

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often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif.).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70%. 80%, 90%, or 95% or more sequence identity when compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes generally means sequence identity of at least 60%, 70%, 80%, 90%, or 95% or more sequence identity.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about  $5^{\circ}$  C. lower than the  $T_m$  for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1° C. to about 20° C. lower than the T<sub>m</sub>, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted 60 by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70%, 80%, 85%, 90%, 95%, or more sequence identity

to a reference sequence over a specified comparison window. Optimal alignment for these purposes can be conducted using the global alignment algorithm of Needleman and Wunsch (1970) supra. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except 10 that residue positions that are not identical may differ by conservative amino acid changes.

The use of the term "nucleotide constructs" herein is not intended to limit the embodiments to nucleotide constructs comprising DNA. Those of ordinary skill in the art will 15 recognize that nucleotide constructs, particularly polynucleotides and oligonucleotides composed of ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides, may also be employed in the methods disclosed herein. The nucleotide constructs, nucleic acids, and nucleo- 20 tide sequences of the embodiments additionally encompass all complementary forms of such constructs, molecules, and sequences. Further, the nucleotide constructs, nucleotide molecules, and nucleotide sequences of the embodiments encompass all nucleotide constructs, molecules, and 25 sequences which can be employed in the methods of the embodiments for transforming plants including, but not limited to, those comprised of deoxyribonucleotides, ribonucleotides, and combinations thereof. Such deoxyribonucleotides and ribonucleotides include both naturally 30 occurring molecules and synthetic analogues. The nucleotide constructs, nucleic acids, and nucleotide sequences of the embodiments also encompass all forms of nucleotide constructs including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop 35 structures, and the like.

A further embodiment relates to a transformed organism such as an organism selected from the group consisting of plant and insect cells, bacteria, yeast, baculoviruses, protozoa, nematodes, and algae. The transformed organism comprises: a DNA molecule of the embodiments, an expression cassette comprising the said DNA molecule, or a vector comprising the said expression cassette, which may be stably incorporated into the genome of the transformed organism.

The sequences of the embodiments are provided in DNA constructs for expression in the organism of interest. The construct will include 5' and 3' regulatory sequences operably linked to a sequence of the embodiments. The term "operably linked" as used herein refers to a functional 50 linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and where necessary 55 to join two protein coding regions, contiguous and in the same reading frame. The construct may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple DNA constructs.

Such a DNA construct is provided with a plurality of restriction sites for insertion of the Cry toxin sequence to be under the transcriptional regulation of the regulatory regions. The DNA construct may additionally contain selectable marker genes.

The DNA construct will include in the 5' to 3' direction of transcription: a transcriptional and translational initiation

region (i.e., a promoter), a DNA sequence of the embodiments, and a transcriptional and translational termination region (i.e., termination region) functional in the organism serving as a host. The transcriptional initiation region (i.e., the promoter) may be native, analogous, foreign or heterologous to the host organism and/or to the sequence of the embodiments. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. The term "foreign" as used herein indicates that the promoter is not found in the native organism into which the promoter is introduced. Where the promoter is "foreign" or "heterologous" to the sequence of the embodiments, it is intended that the promoter is not the native or naturally occurring promoter for the operably linked sequence of the embodiments. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence. Where the promoter is a native or natural sequence, the expression of the operably linked sequence is altered from the wild-type expression, which results in an alteration in phenotype.

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The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous to the promoter, the sequence of interest, the plant host, or any combination thereof).

Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau et al. (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon et al. (1991) *Genes Dev.* 5:141-149; Mogen et al. (1990) *Plant Cell* 2:1261-1272; Munroe et al. (1990) *Gene* 91:151-158; Ballas et al. (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi et al. (1987) *Nucleic Acid Res.* 15:9627-9639.

Where appropriate, a nucleic acid may be optimized for increased expression in the host organism. Thus, where the host organism is a plant, the synthetic nucleic acids can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) Plant Physiol. 92:1-11 for a discussion of host-preferred codon usage. For example, although nucleic acid sequences of the embodiments may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray et al. (1989) Nucleic Acids Res. 17:477-498). Thus, the maizepreferred codon for a particular amino acid may be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants is listed in Table 4 of Murray et al., supra. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Pat. Nos. 5,380,831, and 5,436,391, and Murray et al. (1989) Nucleic Acids Res. 17:477-498, herein incorporated by ref-

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other well-characterized sequences that may be deleterious to gene expression. The GC content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. The term "host cell" as used herein refers to a cell which contains a vector and supports the replication and/or expression of the expression vector is intended. Host cells may be

prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells, or monocotyledonous or dicotyledonous plant cells. An example of a monocotyledonous host cell is a maize host cell. When possible, the sequence is modified to avoid predicted hairpin <sup>5</sup> secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein et al. (1989) Proc. Natl. Acad. Sci. USA 86: 6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Gallie et al. (1995) *Gene* 165(2): 233-238), MDMV <sub>15</sub> leader (Maize Dwarf Mosaic Virus), human immunoglobulin heavy-chain binding protein (BiP) (Macejak et al. (1991) Nature 353: 90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling et al. (1987) Nature 325: 622-625); tobacco mosaic virus 20 leader (TMV) (Gallie et al. (1989) in Molecular Biology of RNA, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel et al. (1991) Virology 81: 382-385). See also, Della-Cioppa et al. (1987) Plant Physiol. 84: 965-968.

In preparing the expression cassette, the various DNA fragments may be manipulated so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other 30 manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, in vitro mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved. 35

A number of promoters can be used in the practice of the embodiments. The promoters can be selected based on the desired outcome. The nucleic acids can be combined with constitutive, tissue-preferred, inducible, or other promoters for expression in the host organism. Suitable constitutive 40 promoters for use in a plant host cell include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Pat. No. 6,072,050; the core CaMV 35S promoter (Odell et al. (1985) Nature 313: 810-812); rice actin (McElroy et al. 45 (1990) Plant Cell 2: 163-171); ubiquitin (Christensen et al. (1989) Plant Mol. Biol. 12: 619-632 and Christensen et al. (1992) Plant Mol. Biol. 18: 675-689); pEMU (Last et al. (1991) Theor. Appl. Genet. 81: 581-588); MAS (Velten et al. (1984) EMBO J. 3:2723-2730); ALS promoter (U.S. Pat. 50 No. 5,659,026), and the like. Other constitutive promoters include, for example, those discussed in U.S. Pat. Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611.

Depending on the desired outcome, it may be beneficial to 55 express the gene from an inducible promoter. Of particular interest for regulating the expression of the nucleotide sequences of the embodiments in plants are wound-inducible promoters. Such wound-inducible promoters, may respond to damage caused by insect feeding, and include 60 potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath.* 28: 425-449; Duan et al. (1996) *Nature Biotechnology* 14: 494-498); wun1 and wun2, U.S. Pat. No. 5,428,148; win1 and win2 (Stanford et al. (1989) *Mol. Gen. Genet.* 215: 200-208); systemin (McGurl et al. (1992) *Science* 225: 1570-1573); WIP1 (Rohmeier et al. (1993) *Plant Mol. Biol.* 22: 783-792; Eckelkamp et al. (1993) *FEBS* 

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Letters 323: 73-76); MPI gene (Corderok et al. (1994) *Plant J.* 6(2): 141-150); and the like, herein incorporated by reference.

Additionally, pathogen-inducible promoters may be employed in the methods and nucleotide constructs of the embodiments. Such pathogen-inducible promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi et al. (1983) *Neth. J. Plant Pathol.* 89: 245-254; Uknes et al. (1992) *Plant Cell* 4: 645-656; and Van Loon (1985) *Plant Mol. Virol.* 4: 111-116. See also WO 99/43819, herein incorporated by reference.

Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau et al. (1987) Plant Mol. Biol. 9:335-342; Matton et al. (1989) Molecular Plant-Microbe Interactions 2:325-331; Somsisch et al. (1986) Proc. Natl. Acad. Sci. USA 83:2427-2430; Somsisch et al. (1988) Mol. Gen. Genet. 2:93-98; and Yang (1996) Proc. Natl. Acad. Sci. USA 93:14972-14977. See also, Chen et al. (1996) Plant J. 10:955-966; Zhang et al. (1994) Proc. Natl. Acad. Sci. USA 91:2507-2511; Warner et al. (1993) Plant J. 3:191-201; Siebertz et al. (1989) Plant Cell 1:961-968; U.S. Pat. No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen Fusarium moniliforme (see, for example, Cordero et al. (1992) Physiol. Mol. Plant Path. 41:189-200).

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemicalinducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena et al. (1991) Proc. Natl. Acad. Sci. USA 88:10421-10425 and McNellis et al. (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz et al. (1991) Mol. Gen. Genet. 227:229-237, and U.S. Pat. Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced pesticidal protein expression within a particular plant tissue. Tissue-preferred promoters include those discussed in Yamamoto et al. (1997) Plant J. 12(2)255-265; Kawamata et al. (1997) Plant Cell Physiol. 38(7):792-803; Hansen et al. (1997) Mol. Gen Genet. 254(3):337-343; Russell et al. (1997) Transgenic Res. 6(2):157-168; Rinehart et al. (1996) Plant Physiol. 112(3):1331-1341; Van Camp et al. (1996) Plant Physiol. 112(2):525-535; Canevascini et al. (1996) Plant Physiol. 112(2):513-524; Yamamoto et al. (1994) Plant Cell Physiol. 35(5):773-778; Lam (1994) Results Probl. Cell Differ. 20:181-196; Orozco et al. (1993) Plant Mol Biol. 23(6):1129-1138; Matsuoka et al. (1993) Proc Natl. Acad. Sci. USA 90(20):9586-9590; and Guevara-Garcia et al. (1993) Plant J. 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

Leaf-preferred promoters are known in the art. See, for example, Yamamoto et al. (1997) *Plant J.* 12(2):255-265; Kwon et al. (1994) *Plant Physiol.* 105:357-67; Yamamoto et al. (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor et al. (1993) *Plant J.* 3:509-18; Orozco et al. (1993) *Plant Mol.* 5 *Biol.* 23(6):1129-1138; and Matsuoka et al. (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

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Root-preferred or root-specific promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, 10 for example, Hire et al. (1992) Plant Mol. Biol. 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) Plant Cell 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger et al. (1990) Plant Mol. Biol. 14(3):433-443 15 (root-specific promoter of the mannopine synthase (MAS) gene of Agrobacterium tumefaciens); and Miao et al. (1991) Plant Cell 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz et al. 20 (1990) Plant Cell 2(7):633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume Parasponia andersonii and the related non-nitrogen-fixing nonlegume Trema tomentosa are described. The promoters of these genes were linked to a 25 3-glucuronidase reporter gene and introduced into both the nonlegume Nicotiana tabacum and the legume Lotus corniculatus, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed roIC and 30 roID root-inducing genes of Agrobacterium rhizogenes (see Plant Science (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri et al. (1989) used gene fusion to lacZ to show that the Agrobacterium T-DNA gene 35 encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see EMBO J. 8(2): 40 343-350). The TR1' gene fused to nptII (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster et al. (1995) Plant Mol. Biol. 29(4):759-772); and roIB promoter (Capana et al. (1994) Plant Mol. 45 Biol. 25(4):681-691. See also U.S. Pat. Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development 50 such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson et al. (1989) BioEssays 10:108, herein incorporated by reference. Such seedpreferred promoters include, but are not limited to, Cim1 55 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); and milps (myo-inositol-1-phosphate synthase) (see U.S. Pat. No. 6,225,529, herein incorporated by reference). Gamma-zein and Glob-1 are endosperm-specific promoters. For dicots, seed-specific promoters include, but are not 60 limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, g-zein, waxy, shrunken 1, shrunken 2, globulin 1, etc. See also WO 00/12733, where 65 seed-preferred promoters from end1 and end2 genes are disclosed; herein incorporated by reference. A promoter that

has "preferred" expression in a particular tissue is expressed in that tissue to a greater degree than in at least one other plant tissue. Some tissue-preferred promoters show expression almost exclusively in the particular tissue.

Where low level expression is desired, weak promoters will be used. Generally, the term "weak promoter" as used herein refers to a promoter that drives expression of a coding sequence at a low level. By low level expression at levels of about 1/1000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts is intended. Alternatively, it is recognized that the term "weak promoters" also encompasses promoters that drive expression in only a few cells and not in others to give a total low level of expression. Where a promoter drives expression at unacceptably high levels, portions of the promoter sequence can be deleted or modified to decrease expression levels.

Such weak constitutive promoters include, for example the core promoter of the Rsyn7 promoter (WO 99/43838 and U.S. Pat. No. 6,072,050), the core 35S CaMV promoter, and the like. Other constitutive promoters include, for example, those disclosed in U.S. Pat. Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611; herein incorporated by reference.

Generally, the expression cassette will comprise a select-

able marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). Additional examples of suitable selectable marker genes include, but are not limited to, genes encoding resistance to chloramphenicol (Herrera Estrella et al. (1983) EMBO J. 2:987-992); methotrexate (Herrera Estrella et al. (1983) Nature 303:209-213; and Meijer et al. (1991) Plant Mol. Biol. 16:807-820); streptomycin (Jones et al. (1987) Mol. Gen. Genet. 210:86-91); spectinomycin (Bretagne-Sagnard et al. (1996) Transgenic Res. 5:131-137); bleomycin (Hille et al. (1990) Plant Mol. Biol. 7:171-176); sulfonamide (Guerineau et al. (1990) Plant Mol. Biol. 15:127-136); bromoxynil (Stalker et al. (1988) Science 242:419-423); glyphosate (Shaw et al. (1986) Science 233:478-481; and U.S. Pat. Nos. 7,709,702; and 7,462,481); phosphinothricin (DeBlock et al. (1987) EMBO J. 6:2513-2518). See generally, Yarranton (1992) Curr. Opin. Biotech. 3: 506-511; Christopherson et al. (1992) Proc. Natl. Acad. Sci. USA 89: 6314-6318; Yao et al. (1992) Cell 71: 63-72; Reznikoff (1992) Mol. Microbiol. 6: 2419-2422; Barkley et al. (1980) in The Operon, pp. 177-220; Hu et al. (1987) Cell 48: 555-566; Brown et al. (1987) Cell 49: 603-612; Figge et al. (1988) Cell 52: 713-722; Deuschle et al. (1989) Proc. Natl. Acad. Sci. USA 86: 5400-5404; Fuerst et al. (1989) Proc. Natl. Acad. Sci. USA 86: 2549-2553; Deuschle et al. (1990) Science 248: 480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines et al. (1993) Proc. Natl. Acad. Sci. USA 90: 1917-1921; Labow et al. (1990) Mol. Cell. Biol. 10: 3343-3356; Zambretti et al. (1992) Proc. Natl. Acad. Sci. USA 89: 3952-3956; Bairn et al. (1991) Proc. Natl. Acad. Sci. USA 88: 5072-5076; Wyborski et al. (1991) Nucleic Acids Res. 19: 4647-4653; Hillenand-Wissman (1989) Topics Mol. Struc. Biol. 10: 143-162; Degenkolb et al. (1991) Antimicrob. Agents Chemother. 35: 1591-1595; Kleinschnidt et al. (1988) Biochemistry 27: 1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen et al. (1992) Proc. Natl. Acad. Sci. USA 89: 5547-5551; Oliva et al.

(1992) Antimicrob. Agents Chemother. 36: 913-919; Hlavka et al. (1985) Handbook of Experimental Pharmacology, Vol. 78 (Springer-Verlag, Berlin); and Gill et al. (1988) Nature 334: 721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the embodiments.

The methods of the embodiments involve introducing a polypeptide or polynucleotide into a plant. "Introducing" is 10 intended to mean presenting to the plant the polynucleotide or polypeptide in such a manner that the sequence gains access to the interior of a cell of the plant. The methods of the embodiments do not depend on a particular method for introducing a polynucleotide or polypeptide into a plant, 15 only that the polynucleotide or polypeptides gains access to the interior of at least one cell of the plant. Methods for introducing polynucleotide or polypeptides into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and 20 virus-mediated methods.

"Stable transformation" is intended to mean that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by the progeny thereof. "Transient transformation" is intended 25 to mean that a polynucleotide is introduced into the plant and does not integrate into the genome of the plant or a polypeptide is introduced into a plant.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending 30 on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway et al. (1986) Biotechniques 4: 320-334), electroporation 35 (Riggs et al. (1986) Proc. Natl. Acad. Sci. USA 83: 5602-5606), Agrobacterium-mediated transformation (U.S. Pat. Nos. 5,563,055 and 5,981,840), direct gene transfer (Paszkowski et al. (1984) EMBO J. 3: 2717-2722), and ballistic particle acceleration (see, for example, U.S. Pat. 40 Nos. 4,945,050; 5,879,918; 5,886,244; and U.S. Pat. No. 5,932,782; Tomes et al. (1995) in Plant Cell, Tissue, and Organ Culture: Fundamental Methods, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe et al. (1988) Biotechnology 6: 923-926); and Lec1 transformation (WO 45 00/28058). For potato transformation see Tu et al. (1998) Plant Molecular Biology 37: 829-838 and Chong et al. (2000) Transgenic Research 9: 71-78. Additional transformation procedures can be found in Weissinger et al. (1988) Ann. Rev. Genet. 22: 421-477; Sanford et al. (1987) Par- 50 ticulate Science and Technology 5: 27-37 (onion); Christou et al. (1988) *Plant Physiol.* 87: 671-674 (soybean); McCabe et al. (1988) Bio/Technology 6: 923-926 (soybean); Finer and McMullen (1991) In Vitro Cell Dev. Biol. 27P: 175-182 (soybean); Singh et al. (1998) Theor. Appl. Genet. 96: 55 319-324 (soybean); Datta et al. (1990) Biotechnology 8: 736-740 (rice); Klein et al. (1988) Proc. Natl. Acad. Sci. USA 85: 4305-4309 (maize); Klein et al. (1988) Biotechnology 6:559-563 (maize); U.S. Pat. Nos. 5,240,855; 5,322, 783 and 5,324,646; Klein et al. (1988) Plant Physiol. 91: 60 440-444 (maize); Fromm et al. (1990) Biotechnology 8: 833-839 (maize); Hooykaas-Van Slogteren et al. (1984) Nature (London) 311: 763-764; U.S. Pat. No. 5,736,369 (cereals); Bytebier et al. (1987) Proc. Natl. Acad. Sci. USA 84: 5345-5349 (Liliaceae); De Wet et al. (1985) in The 65 Experimental Manipulation of Ovule Tissues, ed. Chapman et al. (Longman, N.Y.), pp. 197-209 (pollen); Kaeppler et al.

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(1990) Plant Cell Reports 9: 415-418 and Kaeppler et al. (1992) Theor. Appl. Genet. 84: 560-566 (whisker-mediated transformation); D'Halluin et al. (1992) Plant Cell 4: 1495-1505 (electroporation); Li et al. (1993) Plant Cell Reports 12: 250-255 and Christou and Ford (1995) Annals of Botany 75: 407-413 (rice); Osjoda et al. (1996) Nature Biotechnology 14: 745-750 (maize via Agrobacterium tumefaciens); all of which are herein incorporated by reference.

In specific embodiments, the sequences of the embodiments can be provided to a plant using a variety of transient transformation methods. Such transient transformation methods include, but are not limited to, the introduction of the Cry toxin protein or variants and fragments thereof directly into the plant or the introduction of the Cry toxin transcript into the plant. Such methods include, for example, microinjection or particle bombardment. See, for example, Crossway et al. (1986) Mol Gen. Genet. 202: 179-185; Nomura et al. (1986) Plant Sci. 44: 53-58; Hepler et al. (1994) Proc. Natl. Acad. Sci. 91: 2176-2180 and Hush et al. (1994) The Journal of Cell Science 107: 775-784, all of which are herein incorporated by reference. Alternatively, the Cry toxin polynucleotide can be transiently transformed into the plant using techniques known in the art. Such techniques include viral vector system and the precipitation of the polynucleotide in a manner that precludes subsequent release of the DNA. Thus, transcription from the particlebound DNA can occur, but the frequency with which it is released to become integrated into the genome is greatly reduced. Such methods include the use of particles coated with polyethylimine (PEI; Sigma #P3143).

Methods are known in the art for the targeted insertion of a polynucleotide at a specific location in the plant genome. In one embodiment, the insertion of the polynucleotide at a desired genomic location is achieved using a site-specific recombination system. See, for example, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/ 25853, all of which are herein incorporated by reference. Briefly, the polynucleotide of the embodiments can be contained in transfer cassette flanked by two non-identical recombination sites. The transfer cassette is introduced into a plant have stably incorporated into its genome a target site which is flanked by two non-identical recombination sites that correspond to the sites of the transfer cassette. An appropriate recombinase is provided and the transfer cassette is integrated at the target site. The polynucleotide of interest is thereby integrated at a specific chromosomal position in the plant genome.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick et al. (1986) *Plant Cell Reports* 5: 81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive or inducible expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure that expression of the desired phenotypic characteristic has been achieved.

The nucleotide sequences of the embodiments may be provided to the plant by contacting the plant with a virus or viral nucleic acids. Generally, such methods involve incorporating the nucleotide construct of interest within a viral DNA or RNA molecule. It is recognized that the recombinant proteins of the embodiments may be initially synthesized as part of a viral polyprotein, which later may be processed by proteolysis in vivo or in vitro to produce the

desired pesticidal protein. It is also recognized that such a viral polyprotein, comprising at least a portion of the amino acid sequence of a pesticidal protein of the embodiments, may have the desired pesticidal activity. Such viral polyproteins and the nucleotide sequences that encode for them are encompassed by the embodiments. Methods for providing plants with nucleotide constructs and producing the encoded proteins in the plants, which involve viral DNA or RNA molecules are known in the art. See, for example, U.S. Pat. Nos. 5,889,191; 5,889,190; 5,866,785; 5,589,367; and 10 5,316,931; herein incorporated by reference.

The embodiments further relate to plant-propagating material of a transformed plant of the embodiments including, but not limited to, seeds, tubers, corms, bulbs, leaves, and cuttings of roots and shoots.

The embodiments may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plants of interest include, but are not limited to, corn (Zea mays), Brassica sp. (e.g., B. napus, B. rapa, B. iuncea), particularly those Brassica species useful 20 as sources of seed oil, alfalfa (Medicago sativa), rice (Oryza sativa), rye (Secale cereale), sorghum (Sorghum bicolor, Sorghum vulgare), millet (e.g., pearl millet (Pennisetum glaucum), proso millet (Panicum miliaceum), foxtail millet (Setaria italica), finger millet (Eleusine coracana)), sun- 25 flower (Helianthus annuus), safflower (Carthamus tinctorius), wheat (Triticum aestivum), soybean (Glycine max), tobacco (Nicotiana tabacum), potato (Solanum tuberosum), peanuts (Arachis hypogaea), cotton (Gossypium barbadense, Gossypium hirsutum), sweet potato (Ipomoea 30 batatus), cassava (Manihot esculenta), coffee (Coffea spp.), coconut (Cocos nucifera), pineapple (Ananas comosus), citrus trees (Citrus spp.), cocoa (Theobroma cacao), tea (Camellia sinensis), banana (Musa spp.), avocado (Persea americana), fig (Ficus casica), guava (Psidium guajava), 35 mango (Mangifera indica), olive (Olea europaea), papaya (Carica papaya), cashew (Anacardium occidentale), macadamia (Macadamia integrifolia), almond (Prunus amygdalus), sugar beets (Beta vulgaris), sugarcane (Saccharum spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (Lycopersicon esculentum), lettuce (e.g., Lactuca sativa), green beans (Phaseolus vulgaris), lima beans (Phaseolus limensis), peas (Lathyrus spp.), and members of the genus Cucumis such as cucumber (C. sativus), cantaloupe (C. cantalupensis), and musk melon 45 (C. melo). Ornamentals include azalea (Rhododendron spp.), hydrangea (Macrophylla hydrangea), hibiscus (Hibiscus rosasanensis), roses (Rosa spp.), tulips (Tulipa spp.), daffodils (Narcissus spp.), petunias (Petunia hybrida), carnation (Dianthus caryophyllus), poinsettia (Euphorbia pul- 50 cherrima), and chrysanthemum. Conifers that may be employed in practicing the embodiments include, for example, pines such as loblolly pine (Pinus taeda), slash pine (Pinus elliotii), ponderosa pine (Pinus ponderosa), lodgepole pine (Pinus contorta), and Monterey pine (Pinus 55 radiata); Douglas fir (Pseudotsuga menziesii); Western hemlock (Tsuga canadensis); Sitka spruce (Picea glauca); redwood (Sequoia sempervirens); true firs such as silver fir (Abies amabilis) and balsam fir (Abies balsamea); and cedars such as Western red cedar (Thuja plicata) and Alaska 60 yellow-cedar (Chamaecyparis nootkatensis). Plants of the embodiments include crop plants, including, but not limited to: corn, alfalfa, sunflower, Brassica spp., soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, sugarcane, etc.

Turfgrasses include, but are not limited to: annual bluegrass (Poa annua); annual ryegrass (Lolium multiflorum); Canada bluegrass (Poa compressa); Chewings fescue (Festuca rubra); colonial bentgrass (Agrostis tenuis); creeping bentgrass (Agrostis palustris); crested wheatgrass (Agropyron desertorum); fairway wheatgrass (Agropyron cristatum); hard fescue (Festuca longifolia); Kentucky bluegrass (Poa pratensis); orchardgrass (Dactylis glomerate); perennial ryegrass (Lolium perenne); red fescue (Festuca rubra); redtop (Agrostis alba); rough bluegrass (Poa trivialis); sheep fescue (Festuca ovine); smooth bromegrass (Bromus inermis); tall fescue (Festuca arundinacea); timothy (Phleum pratense); velvet bentgrass (Agrostis canina); weeping alkaligrass (Puccinellia distans); western wheatgrass (Agropyron smithii); Bermuda grass (Cynodon spp.); St. Augustine grass (Stenotaphrum secundatum); zoysia grass (Zoysia spp.); Bahia grass (Paspalum notatum); carpet grass (Axonopus affinis); centipede grass (Eremochloa ophiuroides); kikuyu grass (Pennisetum clandesinum); seashore paspalum (Paspalum vaginatum); blue gramma (Bouteloua gracilis); buffalo grass (Buchloe dactyloids); sideoats gramma (Bouteloua curtipendula).

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Plants of interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, millet, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, flax, castor, olive etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mung bean, lima bean, fava bean, lentils, chickpea, etc.

In certain embodiments the nucleic acid sequences of the embodiments can be stacked with any combination of polynucleotide sequences of interest in order to create plants with a desired phenotype. For example, the polynucleotides of the embodiments may be stacked with any other polynucleotides encoding polypeptides having pesticidal and/or insecticidal activity, such as other Bt toxic proteins (described in U.S. Pat. Nos. 5,366,892; 5,747,450; 5,736,514; 5,723,756; 5,593,881; and Geiser et al. (1986) Gene 48:109), pentin (described in U.S. Pat. No. 5,981,722) and 40 the like. The combinations generated can also include multiple copies of any one of the polynucleotides of interest. The polynucleotides of the embodiments can also be stacked with any other gene or combination of genes to produce plants with a variety of desired trait combinations including but not limited to traits desirable for animal feed such as high oil genes (e.g., U.S. Pat. No. 6,232,529); balanced amino acids (e.g. hordothionins (U.S. Pat. Nos. 5,990,389; 5,885,801; 5,885,802; and 5,703,049); barley high lysine (Williamson et al. (1987) Eur. J. Biochem. 165: 99-106; and WO 98/20122) and high methionine proteins (Pedersen et al. (1986) J. Biol. Chem. 261: 6279; Kirihara et al. (1988) Gene 71: 359; and Musumura et al. (1989) Plant Mol. Biol. 12: 123)); increased digestibility (e.g., modified storage proteins (U.S. Pat. No. 6,858,778); and thioredoxins (U.S. Pat. No. 7,009,087), the disclosures of which are herein incorporated by reference.

The polynucleotides of the embodiments can also be stacked with traits desirable for disease or herbicide resistance (e.g., fumonisin detoxification genes (U.S. Pat. No. 5,792,931); avirulence and disease resistance genes (Jones et al. (1994) *Science* 266:789; Martin et al. (1993) *Science* 262: 1432; and Mindrinos et al. (1994) *Cell* 78:1089); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (e.g., bar gene); and glyphosate resistance (EPSPS gene and GAT gene as disclosed in U.S. Pat. Nos. 7,709,702; and 7,462,481; and

traits desirable for processing or process products such as high oil (e.g., U.S. Pat. No. 6,232,529); modified oils (e.g., fatty acid desaturase genes (U.S. Pat. No. 5,952,544; WO 94/11516)); modified starches (e.g., ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching 5 enzymes (SBE) and starch debranching enzymes (SDBE)); and polymers or bioplastics (e.g., U.S. Pat. No. 5,602,321; beta-ketothiolase, polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert et al. (1988) J. Bacteriol. 170: 5837-5847) facilitate expression of polyhydroxy- 10 alkanoates (PHAs)), the disclosures of which are herein incorporated by reference. One could also combine the polynucleotides of the embodiments with polynucleotides providing agronomic traits such as male sterility (e.g., see U.S. Pat. No. 5,583,210), stalk strength, flowering time, or 15 transformation technology traits such as cell cycle regulation or gene targeting (e.g. WO 99/61619; WO 00/17364; WO 99/25821), the disclosures of which are herein incorporated

In some embodiment the stacked trait may be a trait or 20 event that has received regulatory approval including but not limited to the events found at the Center for Environmental Risk Assessment (cera-gmc.org/?action=gm\_crop\_database, which can be accessed using the www prefix) and at the International Service for the Acquisition of Agri-Biotech 25 may be introduced via a suitable vector into a microbial host, isaaa.org/gmapprovaldatabase/default.asp, which can be accessed using the www prefix).

These stacked combinations can be created by any method including but not limited to cross breeding plants by any conventional or TOPCROSS® methodology, or genetic 30 transformation. If the traits are stacked by genetically transforming the plants, the polynucleotide sequences of interest can be combined at any time and in any order. For example, a transgenic plant comprising one or more desired traits can be used as the target to introduce further traits by subsequent 35 transformation. The traits can be introduced simultaneously in a co-transformation protocol with the polynucleotides of interest provided by any combination of transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate transforma- 40 tion cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different promoters. In certain cases, it may be desirable to introduce a transformation cassette that will suppress the expression of the polynucle- 45 otide of interest. This may be combined with any combination of other suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a 50 site-specific recombination system. See, for example, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference.

Compositions of the embodiments find use in protecting 55 plants, seeds, and plant products in a variety of ways. For example, the compositions can be used in a method that involves placing an effective amount of the pesticidal composition in the environment of the pest by a procedure selected from the group consisting of spraying, dusting, 60 broadcasting, or seed coating.

Before plant propagation material (fruit, tuber, bulb, corm, grains, seed), but especially seed, is sold as a commercial product, it is customarily treated with a protectant coating comprising herbicides, insecticides, fungicides, bac- 65 tericides, nematicides, molluscicides, or mixtures of several of these preparations, if desired together with further carri-

ers, surfactants, or application-promoting adjuvants customarily employed in the art of formulation to provide protection against damage caused by bacterial, fungal, or animal pests. In order to treat the seed, the protectant coating may be applied to the seeds either by impregnating the tubers or grains with a liquid formulation or by coating them with a combined wet or dry formulation. In addition, in special cases, other methods of application to plants are possible, e.g., treatment directed at the buds or the fruit.

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The plant seed of the embodiments comprising a nucleotide sequence encoding a pesticidal protein of the embodiments may be treated with a seed protectant coating comprising a seed treatment compound, such as, for example, captan, carboxin, thiram, methalaxyl, pirimiphos-methyl, and others that are commonly used in seed treatment. In one embodiment, a seed protectant coating comprising a pesticidal composition of the embodiments is used alone or in combination with one of the seed protectant coatings customarily used in seed treatment.

It is recognized that the genes encoding the pesticidal proteins can be used to transform insect pathogenic organisms. Such organisms include baculoviruses, fungi, protozoa, bacteria, and nematodes.

A gene encoding a pesticidal protein of the embodiments and said host applied to the environment, or to plants or animals. The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

Microorganism hosts that are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplana) of one or more crops of interest may be selected. These microorganisms are selected so as to be capable of successfully competing in the particular environment with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the pesticidal protein, and desirably, provide for improved protection of the pesticide from environmental degradation and inactiva-

Such microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms such as bacteria, e.g., Pseudomonas, Erwinia, Serratia, Klebsiella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, and Alcaligenes, fungi, particularly yeast, e.g., Saccharomyces, Cryptococcus, Kluvveromyces, Sporobolomyces, Rhodotorula, and Aureobasidium. Of particular interest are such phytosphere bacterial species as Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacteria, Rhodopseudomonas spheroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes entrophus, Clavibacter xyli and Azotobacter vinelandii and phytosphere yeast species such as Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae, and Aureobasidium pollulans. Of particular interest are the pigmented microorganisms.

A number of ways are available for introducing a gene expressing the pesticidal protein into the microorganism host under conditions that allow for stable maintenance and

tial target pests. Epiphytes, for example, can be grampositive or gram-negative bacteria.

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expression of the gene. For example, expression cassettes can be constructed which include the nucleotide constructs of interest operably linked with the transcriptional and translational regulatory signals for expression of the nucleotide constructs, and a nucleotide sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system that is functional in the host, whereby integration or stable maintenance will occur.

Transcriptional and translational regulatory signals include, but are not limited to, promoters, transcriptional 10 initiation start sites, operators, activators, enhancers, other regulatory elements, ribosomal binding sites, an initiation codon, termination signals, and the like. See, for example, U.S. Pat. Nos. 5,039,523 and 4,853,331; EPO 0480762A2; Sambrook; Maniatis et al. (Cold Spring Harbor Laboratory 15 Press, Cold Spring Harbor, N.Y.); Davis et al., eds. (1980) Advanced Bacterial Genetics (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and the references cited therein.

Suitable host cells, where the pesticidal protein-contain- 20 ing cells will be treated to prolong the activity of the pesticidal proteins in the cell when the treated cell is applied to the environment of the target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells that do not produce substances toxic to higher organ- 25 isms, such as mammals. However, organisms that produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes 30 and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and gram-positive, include Enterobacteriaceae, such as Escherichia, Erwinia, Shigella, Salmonella, and Proteus; Bacillaceae; Rhizobiaceae, such as Rhizobium; Spirillaceae, such as photobacterium, 35 Zymomonas, Serratia, Aeromonas, Vibrio, Desulfovibrio, Spirillum; Lactobacillaceae; Pseudomonadaceae, such as Pseudomonas and Acetobacter; Azotobacteraceae and Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such 40 as Saccharomyces and Schizosaccharomyces; and Basidiomycetes yeast, such as Rhodotorula, Aureobasidium, Sporobolomyces, and the like.

Characteristics of particular interest in selecting a host cell for purposes of pesticidal protein production include 45 ease of introducing the pesticidal protein gene into the host, availability of expression systems, efficiency of expression, stability of the protein in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities 50 for the pesticide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other considerations include ease of 55 formulation and handling, economics, storage stability, and the like.

Host organisms of particular interest include yeast, such as *Rhodotorula* spp., *Aureobasidium* spp., *Saccharomyces* spp. (such as *S. cerevisiae*), *Sporobolomyces* spp., phylloplane organisms such as *Pseudomonas* spp. (such as *P. aeruginosa*, *P. fluorescens*), *Erwinia* spp., and *Flavobacterium* spp., and other such organisms, including Bt, *E. coli*, *Bacillus subtilis*, and the like.

Genes encoding the pesticidal proteins of the embodiments can be introduced into microorganisms that multiply on plants (epiphytes) to deliver pesticidal proteins to poten-

Root-colonizing bacteria, for example, can be isolated from the plant of interest by methods known in the art. Specifically, a *Bacillus cereus* strain that colonizes roots can be isolated from roots of a plant (see, for example, Handelsman et al. (1991) *Appl. Environ*. Microbiol. 56:713-718). Genes encoding the pesticidal proteins of the embodiments can be introduced into a root-colonizing *Bacillus cereus* by standard methods known in the art.

Genes encoding pesticidal proteins can be introduced, for example, into the root-colonizing *Bacillus* by means of electrotransformation. Specifically, genes encoding the pesticidal proteins can be cloned into a shuttle vector, for example, pHT3101 (Lerecius et al. (1989) *FEMS Microbiol. Letts.* 60: 211-218. The shuttle vector pHT3101 containing the coding sequence for the particular pesticidal protein gene can, for example, be transformed into the root-colonizing *Bacillus* by means of electroporation (Lerecius et al. (1989) *FEMS Microbiol. Letts.* 60: 211-218).

Expression systems can be designed so that pesticidal proteins are secreted outside the cytoplasm of gram-negative bacteria, such as *E. coli*, for example. Advantages of having pesticidal proteins secreted are: (1) avoidance of potential cytotoxic effects of the pesticidal protein expressed; and (2) improvement in the efficiency of purification of the pesticidal protein, including, but not limited to, increased efficiency in the recovery and purification of the protein per volume cell broth and decreased time and/or costs of recovery and purification per unit protein.

Pesticidal proteins can be made to be secreted in *E. coli*, for example, by fusing an appropriate *E. coli* signal peptide to the amino-terminal end of the pesticidal protein. Signal peptides recognized by *E. coli* can be found in proteins already known to be secreted in *E. coli*, for example the OmpA protein (Ghrayeb et al. (1984) *EMBO J*, 3:2437-2442). OmpA is a major protein of the *E. coli* outer membrane, and thus its signal peptide is thought to be efficient in the translocation process. Also, the OmpA signal peptide does not need to be modified before processing as may be the case for other signal peptides, for example lipoprotein signal peptide (Duffaud et al. (1987) *Meth. Enzymol.* 153: 492).

Pesticidal proteins of the embodiments can be fermented in a bacterial host and the resulting bacteria processed and used as a microbial spray in the same manner that Bt strains have been used as insecticidal sprays. In the case of a pesticidal protein(s) that is secreted from Bacillus, the secretion signal is removed or mutated using procedures known in the art. Such mutations and/or deletions prevent secretion of the pesticidal protein(s) into the growth medium during the fermentation process. The pesticidal proteins are retained within the cell, and the cells are then processed to yield the encapsulated pesticidal proteins. Any suitable microorganism can be used for this purpose. Pseudomonas has been used to express Bt toxins as encapsulated proteins and the resulting cells processed and sprayed as an insecticide (Gaertner et al. (1993), in: Advanced Engineered Pesticides, ed. Kim).

Alternatively, the pesticidal proteins are produced by introducing a heterologous gene into a cellular host. Expression of the heterologous gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. These cells are then treated under conditions that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of target pest(s). The resulting product retains the toxicity of the toxin. These

naturally encapsulated pesticidal proteins may then be formulated in accordance with conventional techniques for application to the environment hosting a target pest, e.g., soil, water, and foliage of plants. See, for example EP0192319, and the references cited therein.

In the embodiments, a transformed microorganism (which includes whole organisms, cells, spore(s), pesticidal protein(s), pesticidal component(s), pest-impacting component(s), mutant(s), living or dead cells and cell components, including mixtures of living and dead cells and cell com- 10 ponents, and including broken cells and cell components) or an isolated pesticidal protein can be formulated with an acceptable carrier into a pesticidal composition(s) that is, for example, a suspension, a solution, an emulsion, a dusting powder, a dispersible granule or pellet, a wettable powder, 15 and an emulsifiable concentrate, an aerosol or spray, an impregnated granule, an adjuvant, a coatable paste, a colloid, and also encapsulations in, for example, polymer substances. Such formulated compositions may be prepared by such conventional means as desiccation, lyophilization, 20 homogenization, extraction, filtration, centrifugation, sedimentation, or concentration of a culture of cells comprising the polypeptide.

Such compositions disclosed above may be obtained by the addition of a surface-active agent, an inert carrier, a 25 preservative, a humectant, a feeding stimulant, an attractant, an encapsulating agent, a binder, an emulsifier, a dye, a UV protectant, a buffer, a flow agent or fertilizers, micronutrient donors, or other preparations that influence plant growth. One or more agrochemicals including, but not limited to, 30 herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides, acaricides, plant growth regulators, harvest aids, and fertilizers, can be combined with carriers, surfactants or adjuvants customarily employed in the art of formulation or other components to facilitate product han- 35 dling and application for particular target pests. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, e.g., natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, binders, or 40 fertilizers. The active ingredients of the embodiments are normally applied in the form of compositions and can be applied to the crop area, plant, or seed to be treated. For example, the compositions of the embodiments may be applied to grain in preparation for or during storage in a 45 grain bin or silo, etc. The compositions of the embodiments may be applied simultaneously or in succession with other compounds. Methods of applying an active ingredient of the embodiments or an agrochemical composition of the embodiments that contains at least one of the pesticidal 50 proteins produced by the bacterial strains of the embodiments include, but are not limited to, foliar application, seed coating, and soil application. The number of applications and the rate of application depend on the intensity of infestation by the corresponding pest.

Suitable surface-active agents include, but are not limited to, anionic compounds such as a carboxylate of, for example, a metal; a carboxylate of a long chain fatty acid; an N-acylsarcosinate; mono or di-esters of phosphoric acid with fatty alcohol ethoxylates or salts of such esters; fatty 60 alcohol sulfates such as sodium dodecyl sulfate, sodium octadecyl sulfate or sodium cetyl sulfate; ethoxylated fatty alcohol sulfates; ethoxylated alkylphenol sulfates; lignin sulfonates; petroleum sulfonates; alkyl aryl sulfonates such as alkyl-benzene sulfonates or lower alkylnaphtalene 65 sulfonates, e.g., butyl-naphthalene sulfonate; salts of sulfonated naphthalene-formaldehyde condensates; salts of

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sulfonated phenol-formaldehyde condensates; more complex sulfonates such as the amide sulfonates, e.g., the sulfonated condensation product of oleic acid and N-methyl taurine; or the dialkyl sulfosuccinates, e.g., the sodium sulfonate of dioctyl succinate. Non-ionic agents include condensation products of fatty acid esters, fatty alcohols, fatty acid amides or fatty-alkyl- or alkenyl-substituted phenols with ethylene oxide, fatty esters of polyhydric alcohol ethers, e.g., sorbitan fatty acid esters, condensation products of such esters with ethylene oxide, e.g., polyoxyethylene sorbitar fatty acid esters, block copolymers of ethylene oxide and propylene oxide, acetylenic glycols such as 2,4, 7,9-tetraethyl-5-decyn-4,7-diol, or ethoxylated acetylenic glycols. Examples of a cationic surface-active agent include, for instance, an aliphatic mono-, di-, or polyamine such as an acetate, naphthenate or oleate; or oxygen-containing amine such as an amine oxide of polyoxyethylene alkylamine; an amide-linked amine prepared by the condensation of a carboxylic acid with a di- or polyamine; or a quaternary ammonium salt.

Examples of inert materials include but are not limited to inorganic minerals such as kaolin, phyllosilicates, carbonates, sulfates, phosphates, or botanical materials such as cork, powdered corncobs, peanut hulls, rice hulls, and walnut shells.

The compositions of the embodiments can be in a suitable form for direct application or as a concentrate of primary composition that requires dilution with a suitable quantity of water or other diluent before application. The pesticidal concentration will vary depending upon the nature of the particular formulation, specifically, whether it is a concentrate or to be used directly. The composition contains 1 to 98% of a solid or liquid inert carrier, and 0 to 50% or 0.1 to 50% of a surfactant. These compositions will be administered at the labeled rate for the commercial product, for example, about 0.01 lb-5.0 lb. per acre when in dry form and at about 0.01 pts.-10 pts. per acre when in liquid form.

In a further embodiment, the compositions, as well as the transformed microorganisms and pesticidal proteins of the embodiments, can be treated prior to formulation to prolong the pesticidal activity when applied to the environment of a target pest as long as the pretreatment is not deleterious to the pesticidal activity. Such treatment can be by chemical and/or physical means as long as the treatment does not deleteriously affect the properties of the composition(s). Examples of chemical reagents include but are not limited to halogenating agents; aldehydes such as formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride; alcohols, such as isopropanol and ethanol; and histological fixatives, such as Bouin's fixative and Helly's fixative (see, for example, Humason (1967) *Animal Tissue Techniques* (W.H. Freeman and Co.).

In other embodiments, it may be advantageous to treat the Cry toxin polypeptides with a protease, for example trypsin, to activate the protein prior to application of a pesticidal protein composition of the embodiments to the environment of the target pest. Methods for the activation of protoxin by a serine protease are well known in the art. See, for example, Cooksey (1968) *Biochem. J.* 6:445-454 and Carroll and Ellar (1989) *Biochem. J.* 261:99-105, the teachings of which are herein incorporated by reference. For example, a suitable activation protocol includes, but is not limited to, combining a polypeptide to be activated, for example a purified novel Cry polypeptide (e.g., having the amino acid sequence set forth in SEQ ID NO: 2), and trypsin at a 1/100 weight ratio of protein/trypsin in 20 nM NaHCO<sub>3</sub>, pH 8 and digesting the sample at 36° C. for 3 hours.

The compositions (including the transformed microorganisms and pesticidal proteins of the embodiments) can be applied to the environment of an insect pest by, for example, spraying, atomizing, dusting, scattering, coating or pouring, introducing into or on the soil, introducing into irrigation 5 water, by seed treatment or general application or dusting at the time when the pest has begun to appear or before the appearance of pests as a protective measure. For example, the pesticidal protein and/or transformed microorganisms of the embodiments may be mixed with grain to protect the 10 grain during storage. It is generally important to obtain good control of pests in the early stages of plant growth, as this is the time when the plant can be most severely damaged. The compositions of the embodiments can conveniently contain another insecticide if this is thought necessary. In one 15 embodiment, the composition is applied directly to the soil, at a time of planting, in granular form of a composition of a carrier and dead cells of a Bacillus strain or transformed microorganism of the embodiments. Another embodiment is a granular form of a composition comprising an agrochemi- 20 cal such as, for example, an herbicide, an insecticide, a fertilizer, an inert carrier, and dead cells of a Bacillus strain or transformed microorganism of the embodiments.

Those skilled in the art will recognize that not all compounds are equally effective against all pests. Compounds of 25 the embodiments display activity against insect pests, which may include economically important agronomic, forest, greenhouse, nursery, ornamentals, food and fiber, public and animal health, domestic and commercial structure, household, and stored product pests. Insect pests include insects 30 selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera.

Insects of the order Lepidoptera include, but are not limited to, armyworms, cutworms, loopers, and heliothines in the family Noctuidae Agrotis ipsilon Hufnagel (black cutworm); A. orthogonia Morrison (western cutworm); A. segetum Denis & Schiffermüller (turnip moth); A. subter- 40 ranea Fabricius (granulate cutworm); Alabama argillacea Hübner (cotton leaf worm); Anticarsia gemmatalis Hübner (velvetbean caterpillar); Athetis mindara Barnes and McDunnough (rough skinned cutworm); Earias insulana Boisduval (spiny bollworm); E. vittella Fabricius (spotted 45 bollworm); Egira (Xylomyges) curialis Grote (citrus cutworm); Euxoa messoria Harris (darksided cutworm); Helicoverpa armigera Hübner (American bollworm); H. zea Boddie (corn earworm or cotton bollworm); Heliothis virescens Fabricius (tobacco budworm); Hypena scabra Fab- 50 ricius (green cloverworm); Mamestra configurata Walker (bertha armyworm); M. brassicae Linnaeus (cabbage moth); Melanchra picta Harris (zebra caterpillar); Pseudaletia unipuncta Haworth (armyworm); Pseudoplusia includens Walker (soybean looper); Richia albicosta Smith (Western 55 bean cutworm); Spodoptera frugiperda JE Smith (fall armyworm); S. exigua Hübner (beet armyworm); S. litura Fabricius (tobacco cutworm, cluster caterpillar); Trichoplusia ni Hübner (cabbage looper); borers, casebearers, webworms, coneworms, and skeletonizers from the families Pyralidae 60 and Crambidae such as Achroia grisella Fabricius (lesser wax moth); Amyelois transitella Walker (naval orangeworm); Anagasta kuehniella Zeller (Mediterranean flour moth); Cadra cautella Walker (almond moth); Chilo partellus Swinhoe (spotted stalk borer); C. suppressalis Walker 65 (striped stem/rice borer); C. terrenellus Pagenstecher (sugarcane stemp borer); Corcyra cephalonica Stainton (rice

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moth); Crambus caliginosellus Clemens (corn root webworm); C. teterrellus Zincken (bluegrass webworm); Cnaphalocrocis medinalis Guenee (rice leaf roller); Desmia funeralis Hübner (grape leaffolder); Diaphania hyalinata Linnaeus (melon worm); D. nitidalis Stoll (pickleworm); Diatraea grandiosella Dyar (southwestern corn borer), D. saccharalis Fabricius (surgarcane borer); Elasmopalpus lignosellus Zeller (lesser cornstalk borer); Eoreuma loftini Dyar (Mexican rice borer); Ephestia elutella Hübner (tobacco (cacao) moth); Galleria mellonella Linnaeus (greater wax moth); Hedylepta accepta Butler (sugarcane leafroller); Herpetogramma licarsisalis Walker (sod webworm); Homoeosoma electellum Hulst (sunflower moth); Loxostege sticticalis Linnaeus (beet webworm); Maruca testulalis Gever (bean pod borer); Orthaga thvrisalis Walker (tea tree web moth); Ostrinia nubilalis Hübner (European corn borer); Plodia interpunctella Hübner (Indian meal moth); Scirpophaga incertulas Walker (yellow stem borer); Udea rubigalis Guenee (celery leaftier); and leaf rollers, budworms, seed worms, and fruit worms in the family Tortricidae Acleris gloverana Walsingham (Western blackheaded budworm); A. variana Fernald (Eastern blackheaded budworm); Adoxophyes orana Fischer von Rösslerstamm (summer fruit tortrix moth); Archips spp. including A. argyrospila Walker (fruit tree leaf roller) and A. rosana Linnaeus (European leaf roller); Argyrotaenia spp.; Bonagota salubricola Meyrick (Brazilian apple leafroller); Choristoneura spp.; Cochylis hospes Walsingham (banded sunflower moth); Cvdia latiferreana Walsingham (filbertworm); C. pomonella Linnaeus (codling moth); Endopiza viteana Clemens (grape berry moth); Eupoecilia ambiguella Hübner (vine moth); Grapholita molesta Busck (oriental fruit moth); Lobesia botrana Denis & Schiffermüller (European grape vine moth); Platynota flavedana Clemens (variegated leafroller); 35 P. stultana Walsingham (omnivorous leaf roller); Spilonota ocellana Denis & Schiffermüller (eyespotted bud moth); and Suleima helianthana Riley (sunflower bud moth).

Selected other agronomic pests in the order Lepidoptera include, but are not limited to, Alsophila pometaria Harris (fall cankerworm); Anarsia lineatella Zeller (peach twig borer); Anisota senatoria J. E. Smith (orange striped oakworm); Antheraea pernyi Guërin-Mëneville (Chinese Oak Silkmoth); Bombyx mori Linnaeus (Silkworm); Bucculatrix thurberiella Busck (cotton leaf perforator); Collas eurytheme Boisduval (alfalfa caterpillar); Datana integerrima Grote & Robinson (walnut caterpillar); Dendrolimus sibiricus Tschetwerikov (Siberian silk moth), Ennomos subsignaria Hübner (elm spanworm); Erannis tiliaria Harris (linden looper); Erechthias flavistriata Walsingham (sugarcane bud moth); Euproctis chrysorrhoea Linnaeus (browntail moth); Harrisina americana Guérin-Meneville (grapeleaf skeletonizer); Heliothis subflexa Guenee; Hemileuca oliviae Cockrell (range caterpillar); Hyphantria cunea Drury (fall webworm); Keiferia lycopersicella Walsingham (tomato pinworm); Lambdina fiscellaria fiscellaria Hulst (Eastern hemlock looper); L. fiscellaria lugubrosa Hulst (Western hemlock looper); Leucoma salicis Linnaeus (satin moth); Lymantria dispar Linnaeus (gypsy moth); Malacosoma spp.; Manduca quinquemaculata Haworth (five spotted hawk moth, tomato hornworm); M. sexta Haworth (tomato hornworm, tobacco hornworm); Operophtera brumata Linnaeus (winter moth); Orgyia spp.; Paleacrita vernata Peck (spring cankerworm); Papilio cresphontes Cramer (giant swallowtail, orange dog); Phryganidia californica Packard (California oakworm); Phyllocnistis citrella Stainton (citrus leafminer); Phyllonorycter blancardella Fabricius (spotted tentiform leafminer); Pieris brassicae Linnaeus (large white

butterfly); *P. rapae* Linnaeus (small white butterfly); *P. napi* Linnaeus (green veined white butterfly); *Platyptilia carduidactyla* Riley (artichoke plume moth); *Plutella xylostella* Linnaeus (diamondback moth); *Pectinophora gossypiella* Saunders (pink bollworm); *Pontia protodice* Boisduval & 5 Leconte (Southern cabbageworm); *Sabulodes aegrotata* Guenée (omnivorous looper); *Schizura concinna* J. E. Smith (red humped caterpillar); *Sitotroga cerealella* Olivier (Angoumois grain moth); *Thaumetopoea pityocampa* Schiffermuller (pine processionary caterpillar); *Tineola bisselliella* 10 Hummel (webbing clothesmoth); *Tuta absoluta* Meyrick (tomato leafminer) and *Yponomeuta padella* Linnaeus (ermine moth).

Of interest are larvae and adults of the order Coleoptera including weevils from the families Anthribidae, Bruchidae, 15 and Curculionidae including, but not limited to: Anthonomus grandis Boheman (boll weevil); Cylindrocopturus adspersus LeConte (sunflower stem weevil); Diaprepes abbreviatus Linnaeus (Diaprepes root weevil); Hypera punctata Fabricius (clover leaf weevil): Lissorhoptrus orvzophilus 20 Kuschel (rice water weevil); Metamasius hemipterus hemipterus Linnaeus (West Indian cane weevil); M. hemipterus sericeus Olivier (silky cane weevil); Sitophilus granarius Linnaeus (granary weevil); S. oryzae Linnaeus (rice weevil); Smicronyx fulvus LeConte (red sunflower seed 25 weevil); S. sordidus LeConte (gray sunflower seed weevil); Sphenophorus maidis Chittenden (maize billbug); Rhabdoscelus obscurus Boisduval (New Guinea sugarcane weevil); flea beetles, cucumber beetles, rootworms, leaf beetles, potato beetles, and leafminers in the family Chrysomelidae 30 including, but not limited to: Chaetocnema ectypa Horn (desert corn flea beetle); C. pulicaria Melsheimer (corn flea beetle); Colaspis brunnea Fabricius (grape colaspis); Diabrotica barberi Smith & Lawrence (northern corn rootworm); D. undecimpunctata howardi Barber (southern corn 35 rootworm); D. virgifera virgifera LeConte (western corn rootworm); Leptinotarsa decemlineata Say (Colorado potato beetle); Oulema melanopus Linnaeus (cereal leaf beetle); Phyllotreta cruciferae Goeze (corn flea beetle); Zygogramma exclamationis Fabricius (sunflower beetle); 40 beetles from the family Coccinellidae including, but not limited to: Epilachna varivestis Mulsant (Mexican bean beetle); chafers and other beetles from the family Scarabaeidae including, but not limited to: Antitrogus parvulus Britton (Childers cane grub); Cyclocephala borealis Arrow (north- 45 ern masked chafer, white grub); C. immaculata Olivier (southern masked chafer, white grub); Dermolepida albohirtum Waterhouse (Greyback cane beetle); Euetheola humilis rugiceps LeConte (sugarcane beetle); Lepidiota frenchi Blackburn (French's cane grub); Tomarus gibbosus De Geer 50 (carrot beetle); T. subtropicus Blatchley (sugarcane grub); Phyllophaga crinita Burmeister (white grub); P. latifrons LeConte (June beetle); Popillia japonica Newman (Japanese beetle); Rhizotrogus majalis Razoumowsky (European chafer); carpet beetles from the family Dermestidae; wire- 55 worms from the family Elateridae, Eleodes spp., Melanotus spp. including *M. communis* Gyllenhal (wireworm); Conoderus spp.; Limonius spp.; Agriotes spp.; Ctenicera spp.; Aeolus spp.; bark beetles from the family Scolytidae; beetles from the family Tenebrionidae; beetles from the 60 family Cerambycidae such as, but not limited to, Migdolus fryanus Westwood (longhorn beetle); and beetles from the Buprestidae family including, but not limited to, Aphanisticus cochinchinae seminulum Obenberger (leaf-mining buprestid beetle).

Adults and immatures of the order Diptera are of interest, including leafminers Agromyza parvicornis Loew (corn

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blotch leafminer); midges including, but not limited to: Contarinia sorghicola Coquillett (sorghum midge); Mayetiola destructor Say (Hessian fly); Neolasioptera murtfeldtiana Felt, (sunflower seed midge); Sitodiplosis mosellana Géhin (wheat midge); fruit flies (Tephritidae), Oscinella frit Linnaeus (frit flies); maggots including, but not limited to: Delia spp. including Delia platura Meigen (seedcorn maggot); D. coarctata Fallen (wheat bulb fly); Fannia canicularis Linnaeus, F. femoralis Stein (lesser house flies); Meromyza americana Fitch (wheat stem maggot); Musca domestica Linnaeus (house flies); Stomoxys calcitrans Linnaeus (stable flies)); face flies, horn flies, blow flies, Chrysomya spp.; Phormia spp.; and other muscoid fly pests, horse flies Tabanus spp.; bot flies Gastrophilus spp.; Oestrus spp.; cattle grubs *Hypoderma* spp.; deer flies *Chrysops* spp.; Melophagus ovinus Linnaeus (keds); and other Brachycera, mosquitoes Aedes spp.; Anopheles spp.; Culex spp.; black flies Prosimulium spp.; Simulium spp.; biting midges, sand flies, sciarids, and other Nematocera.

Included as insects of interest are those of the order Hemiptera such as, but not limited to, the following families: Adelgidae, Aleyrodidae, Aphididae, Asterolecaniidae, Cercopidae, Cicadellidae, Cicadidae, Cixiidae, Coccidae, Coreidae, Dactylopiidae, Delphacidae, Diaspididae, Eriococcidae, Flatidae, Fulgoridae, Issidae, Lygaeidae, Margarodidae, Membracidae, Miridae, Ortheziidae, Pentatomidae, Phoenicococcidae, Phylloxeridae, Pseudococcidae, Psyllidae, Pyrrhocoridae and Tingidae.

Agronomically important members from the order Hemiptera include, but are not limited to: Acrosternum hilare Say (green stink bug); Acyrthisiphon pisum Harris (pea aphid); Adelges spp. (adelgids); Adelphocoris rapidus Say (rapid plant bug); Anasa tristis De Geer (squash bug); Aphis craccivora Koch (cowpea aphid); A. fabae Scopoli (black bean aphid); A. gossypii Glover (cotton aphid, melon aphid); A. maidiradicis Forbes (corn root aphid); A. pomi De Geer (apple aphid); A. spiraecola Patch (spirea aphid); Aulacaspis tegalensis Zehntner (sugarcane scale); Aulacorthum solani Kaltenbach (foxglove aphid); Bemisia tabaci Gennadius (tobacco whitefly, sweetpotato whitefly); B. argentifolii Bellows & Perring (silverleaf whitefly); Blissus leucopterus leucopterus Say (chinch bug); Blostomatidae spp.; Brevicoryne brassicae Linnaeus (cabbage aphid); Cacopsylla pyricola Foerster (pear psylla); Calocoris norvegicus Gmelin (potato capsid bug); Chaetosiphon fragaefolii Cockerell (strawberry aphid); Cimicidae spp.; Coreidae spp.; Corythuca gossypii Fabricius (cotton lace bug); Cyrtopeltis modesta Distant (tomato bug); C. notatus Distant (suckfly); Deois flavopicta Stål (spittlebug); Dialeurodes citri Ashmead (citrus whitefly); Diaphnocoris chlorionis Say (honeylocust plant bug); Diuraphis noxia Kurdjumov/ Mordvilko (Russian wheat aphid); Duplachionaspis divergens Green (armored scale); Dysaphis plantaginea Paaserini (rosy apple aphid); Dysdercus suturellus Herrich-Schaffer (cotton stainer); Dysmicoccus boninsis Kuwana (gray sugarcane mealybug); Empoasca fabae Harris (potato leafhopper); Eriosoma lanigerum Hausmann (woolly apple aphid); Erythroneoura spp. (grape leafhoppers); Eumetopina flavipes Muir (Island sugarcane planthopper); Eurygaster spp.; Euschistus servus Say (brown stink bug); E. variolarius Palisot de Beauvois (one-spotted stink bug); Graptostethus spp. (complex of seed bugs); and Hyalopterus pruni Geoffroy (mealy plum aphid); Icerva purchasi Maskell (cottony cushion scale); Labopidicola allii Knight (onion plant bug); Laodelphax striatellus Fallen (smaller brown planthopper); Leptoglossus corculus Say (leaf-footed pine seed bug);

Leptodictya tabida Herrich-Schaeffer (sugarcane lace bug);

Lipaphis erysimi Kaltenbach (turnip aphid); Lygocoris pabulinus Linnaeus (common green capsid); Lygus lineolaris Palisot de Beauvois (tarnished plant bug); L. Hesperus Knight (Western tarnished plant bug); L. pratensis Linnaeus (common meadow bug); L. rugulipennis Poppius (European tarnished plant bug); Macrosiphum euphorbiae Thomas (potato aphid); Macrosteles quadrilineatus Forbes (aster leafhopper); Magicicada septendecim Linnaeus (periodical cicada); Mahanarva fimbriolata Stål (sugarcane spittlebug); Melanaphis sacchari Zehntner (sugarcane 10 aphid); Melanaspis glomerata Green (black scale); Metopolophium dirhodum Walker (rose grain aphid); Myzus persicae Sulzer (peach-potato aphid, green peach aphid); Nasonovia ribisnigri Mosley (lettuce aphid); Nephotettix cinticeps Uhler (green leafhopper); N. nigropictus Stål (rice 15 leafhopper); Nezara viridula Linnaeus (southern green stink bug); Nilaparvata lugens Stål (brown planthopper); Nysius ericae Schilling (false chinch bug); Nysius raphanus Howard (false chinch bug); Oebalus pugnax Fabricius (rice stink bug); Oncopeltus fasciatus Dallas (large milkweed bug); 20 Orthops campestris Linnaeus; Pemphigus spp. (root aphids and gall aphids); Peregrinus maidis Ashmead (corn planthopper); Perkinsiella saccharicida Kirkaldy (sugarcane delphacid); Phylloxera devastatrix Pergande (pecan phylloxera); Planococcus citri Risso (citrus mealybug); Plesio- 25 coris rugicollis Fallen (apple capsid); Poecilocapsus lineatus Fabricius (four-lined plant bug); Pseudatomoscelis seriatus Reuter (cotton fleahopper); Pseudococcus spp. (other mealybug complex); Pulvinaria elongata Newstead (cottony grass scale); Pyrilla perpusilla Walker (sugarcane 30 leafhopper); Pyrrhocoridae spp.; Quadraspidiotus perniciosus Comstock (San Jose scale); Reduviidae spp.; Rhopalosiphum maidis Fitch (corn leaf aphid); R. padi Linnaeus (bird cherry-oat aphid); Saccharicoccus sacchari Cockerell (pink sugarcane mealybug); Schizaphis graminum Rondani 35 (greenbug); Sipha flava Forbes (yellow sugarcane aphid); Sitobion avenae Fabricius (English grain aphid); Sogatella furcifera Horvath (white-backed planthopper); Sogatodes oryzicola Muir (rice delphacid); Spanagonicus albofasciatus Reuter (whitemarked fleahopper); Therioaphis maculata 40 Buckton (spotted alfalfa aphid); Tinidae spp.; Toxoptera aurantii Boyer de Fonscolombe (black citrus aphid); and T. citricida Kirkaldy (brown citrus aphid); Trialeurodes abutiloneus (bandedwinged whitefly) and T. vaporariorum Westwood (greenhouse whitefly); Trioza diospyri Ashmead 45 (persimmon psylla); and Typhlocyba pomaria McAtee (white apple leafhopper).

Also included are adults and larvae of the order Acari (mites) such as Aceria tosichella Keifer (wheat curl mite); Panonychus ulmi Koch (European red mite); Petrobia latens 50 Müller (brown wheat mite); Steneotarsonemus bancrofti Michael (sugarcane stalk mite); spider mites and red mites in the family Tetranychidae, Oligonychus grypus Baker & Pritchard, O. indicus Hirst (sugarcane leaf mite), O. pratensis Banks (Banks grass mite), O. stickneyi McGregor 55 (sugarcane spider mite); Tetranychus urticae Koch (two spotted spider mite); T. mcdanieli McGregor (McDaniel mite); T. cinnabarinus Boisduval (carmine spider mite); T. turkestani Ugarov & Nikolski (strawberry spider mite), flat mites in the family Tenuipalpidae, Brevipalpus lewisi 60 McGregor (citrus flat mite); rust and bud mites in the family Eriophyidae and other foliar feeding mites and mites important in human and animal health, i.e. dust mites in the family Epidermoptidae, follicle mites in the family Demodicidae, grain mites in the family Glycyphagidae, ticks in the order 65 Ixodidae. Ixodes scapularis Say (deer tick); I. holocyclus Neumann (Australian paralysis tick); Dermacentor variabi48

lis Say (American dog tick); *Amblyomma americanum* Linnaeus (lone star tick); and scab and itch mites in the families Psoroptidae, Pyemotidae, and Sarcoptidae.

Insect pests of the order Thysanura are of interest, such as *Lepisma saccharina* Linnaeus (silverfish); *Thermobia domestica* Packard (firebrat).

Additional arthropod pests covered include: spiders in the order Araneae such as *Loxosceles reclusa* Gertsch & Mulaik (brown recluse spider); and the *Latrodectus mactans* Fabricius (black widow spider); and centipedes in the order Scutigeromorpha such as *Scutigera coleoptrata* Linnaeus (house centipede). In addition, insect pests of the order Isoptera are of interest, including those of the termitidae family, such as, but not limited to, *Cylindrotermes nordenskioeldi* Holmgren and *Pseudacanthotermes militaris* Hagen (sugarcane termite). Insects of the order Thysanoptera are also of interest, including but not limited to thrips, such as *Stenchaetothrips minutus* van Deventer (sugarcane thrips).

Insect pests may be tested for pesticidal activity of compositions of the embodiments in early developmental stages, e.g., as larvae or other immature forms. The insects may be reared in total darkness at from about 20° C. to about 30° C. and from about 30% to about 70% relative humidity. Bioassays may be performed as described in Czapla and Lang (1990) *J. Econ. Entomol.* 83(6): 2480-2485. Methods of rearing insect larvae and performing bioassays are well known to one of ordinary skill in the art.

A wide variety of bioassay techniques are known to one skilled in the art. General procedures include addition of the experimental compound or organism to the diet source in an enclosed container. Pesticidal activity can be measured by, but is not limited to, changes in mortality, weight loss, attraction, repellency and other behavioral and physical changes after feeding and exposure for an appropriate length of time. Bioassays described herein can be used with any feeding insect pest in the larval or adult stage.

The following examples are presented by way of illustration, not by way of limitation.

### **EXPERIMENTALS**

# Example 1—Gene Identification and *E. coli* Expression

The polynucleotide of SEQ ID NO: 1 encoding insecticidal protein (SEQ ID NO: 2) designated as MP372, was obtained from a Bacillus thuringiensis isolate designated as DP250 from an internal DuPont proprietary collection of Bacillus thuringiensis isolates. The polynucleotide (SEQ ID NO: 1) encoding the insecticidal polypeptide (SEQ ID NO: 2) was cloned into a pET28a vector (Novagen®) and transformed into E. coli BL21 cells (Invitrogen). Large scale 1.0 L cultures were grown until O.D. 600 nm~0.8 and then the cultures were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) 1 mM and allowed to grow for 16 hours at 16° C. The cell pellets were lysed with 50 mL of 500 mM NaCl/20 mM Tris/5 mM Imidazole/pH 7.9 with 0.02% lysozyme (w/v) and 0.1% Tween-20 and 1 tablet of Complete Protease Inhibitor (Roche) added. After lysis, the solutions were sonicated and the lysate centrifuged at 25,000 rpm for 30 minutes. The supernatant containing the soluble protein fraction were filtered through a 0.45 u vacuum filter and then 1 ml of Talon (Clontech) slurry is added and then incubated for binding on rotator at 100 rpm for 1 hour. The lysate is then added to a column and the bound protein is isolated and washed with 20 ml of 50 mM NaCl/20 mM

Tris/5 mM Imidazole/pH 7.9 and then eluted with 1.5 ml of 50 mmM NaCl/20 mM Tris/500 mM Imidazole/pH 7.9. The purified protein is then dialyzed into 50 mM sodium carbonate buffer pH10. The purified protein was submitted for insecticidal activity in panel of Lepidoptera and Coleoptera in vitro feeding assays. The insecticidal activity for MP372 (SEQ ID NO: 4) is shown in Table 1.

### Example 2—Cyr1Ea/MP327 Chimera

A chimera was designed with Domain I (amino acids 1-259 of SEQ ID NO: 6) from Cry1Ea (Accession No. X53985) and Domain II and Domain III (amino acids 259-617 of SEQ ID NO: 2) from MP372. The resulting chimera was designated as MP327-Mut 5 having the polynucleotide sequence of SEQ ID NO: 3 encoding the polypeptide of SEQ ID NO: 4. The purified protein was submitted for insecticidal activity against a panel of Lepidoptera and Coleoptera species in vitro feeding assays. The insecticidal activity for MP372 (SEQ ID NO: 4) is shown in Table 20 1. The MP372-Mut 5 (SEQ ID NO: 4) variant has a broader insecticidal spectrum by introducing FAW activity and improves CEW, SBL and VBC efficacies compared to MP327 (SEQ ID NO: 2).

# Example 3—Insecticidal Assays with Purified Proteins

Insecticidal activity bioassay screens were conducted to evaluate the effects of the insecticidal proteins on a variety of Lepidoptera species: European corn borer (Ostrinia nubilalis); corn earworm (Helicoverpa zea); black cutworm (Agrotis ipsilon); fall armyworm (Spodoptera frugiperda); Soybean looper (Pseudoplusia includens); and Velvet bean caterpillar (Anticarsia gemmatalis), and a Coleoptera specie 35 (Western corn rootworm (Diabrotica virgifera).

Lepidoptera feeding assays were conducted on an artificial diet containing the cleared lysates of bacterial strains in a 96 well plate set up. The cleared lysate was incorporated with the Lepidopteran-specific artificial diet in a ratio of 20 40 ul cleared lysate and 40 ul of diet mixture. Two to five neonate larvas were placed in each well to feed ad libitum for 5 days. Results were expressed as positive for larvae reactions such as stunting and or mortality. Results were expressed as negative if the larvae were similar to the 45 negative control that is feeding diet to which the above buffer only has been applied. Each cleared lysate was assayed on European corn borer (Ostrinia nubilalis), corn earworm (Helicoverpa zea), black cutworm (Agrotis ipsilon), fall armyworm (Spodoptera frugiperda), Soybean 50 looper (Pseudoplusia includens) and Velvet bean caterpillar (Anticarsia gemmatalis). Larvae were scored for mortality or severity of stunting. The scores were recorded numerically as dead (3), severely stunted (2) (little or no growth but alive and equivalent to a  $1^{st}$  instar larvae), stunted (1) 55 (growth to second instar but not equivalent to controls), or normal (0). The results are shown in Table 1.

TABLE 1

Primary Screen	Concentration (ug/cm2)	ECB	FAW	CEW	SBL	VBC
MP372-Mut 5 (SEQ ID NO: 4)	20	0	3	1	3	3
MP372-WT (SEQ ID NO: 2)	20	0	0	0.75	2	2

For MP372-Mut 5 (SEQ ID NO: 4) a series of concentrations of the purified protein sample was assayed against SBL and VBC, and concentrations for 50% mortality (LC50) or inhibition of 50% of the individuals (1050) were calculated. The results are shown in Table 2.

TABLE 2

Insect	LC/IC	ppm, 4d	Lower 95% CL	Upper 95% CL	Slope
SBL	LC50	1.28	0.28	2.73	4.24
VBC	LC50	1.79	0.50	4.25	2.99

Example 4—Transient Expression and Insect Bioassay on Transient Maize Leaf Tissues

Two gene designs encoding MP372 (SEQ ID NO: 2) were each cloned into a transient expression vector under control of a duplicated version of the promoter from the mirabilis mosaic virus (DMMV PRO; Dey and Maiti, (1999) Plant Mol. Biol., 40:771-82). The agro-infiltration method of introducing an Agrobacterium cell suspension to plant cells of intact tissues so that reproducible infection and subsequent plant derived transgene expression may be measured or studied is well known in the art (Kapila, et. al., (1997) Plant Science 122:101-108). Briefly, young plant disks of maize were agro-infiltrated with normalized bacterial cell cultures of test and control strains. Leaf discs are infested with Soybean Looper (SBL) (Chrysodeixis includens) or Fall Armyworm (FAW) (Spodoptera frugiperda), along with appropriate controls. The degree of consumption of green leaf tissues is scored after 2 days of infestation.

After 2d feeding, the amount of leaf tissue consumed by SBL or FAW larvae is scored across 12 disks per treatment. When compared to negative control, tissue accumulating MP372 is significantly less damaged across the 12 disks for both FAW and SBL (Table 3).

TABLE 3

Gene design	FAW	SBL	
MP372 V1	+	+	
MP372 V2	+	+	

Example 5—Transient Expression and Insect Bioassay on Transient Bush Bean Leaf Tissues

To confirm activity of the insecticidal polypeptides of the disclosure a bush bean transient expression system under control of the AtUBQ10 promoter (Day, et. al., (1999) Plant Mol. Biol. 40:771-782; Norris S R et al (1993) Plant Mol Biol. 21(5):895-906) was utilized. The agro-infiltration method of introducing an Agrobacterium cell suspension to plant cells of intact tissues so that reproducible infection and subsequent plant derived transgene expression may be measured or studied is well known in the art (Kapila, et. al., (1997) Plant Science 122:101-108). Briefly, bush bean 60 (Phaseolus vulgaris) seedlings were agro-infiltrated with normalized bacterial cell cultures of transformed with one of two gene designs encoding MP372 (SEQ ID NO: 2) or MP372 Mut 5 (SEQ ID NO: 4) and control vectors. After 4 days leaf disks were harvested and infested with neonates of Soybean Looper (SBL) (Chrysodeixis includens), Velvetbean Caterpillar (VBC) (Anticarsia gemmatalis), or Fall Armyworm (Spodoptera frugiperda) alone. Control leaf

discs were generated with plants infiltrated with a buffer control. The consumption of green leaf tissue was evaluated two days after infestation (Table 4).

TABLE 4

Gene design	FAW	SBL	VBC	
MP372 V1	_	+	+	
MP372 V2	_	+	+	
MP372 Mut 5 V1	+	+	+	
MP372 Mut 5 V2	+	+	+	

Example 6—Agrobacterium-Mediated Transformation of Maize and Regeneration of Transgenic Plants

For Agrobacterium-mediated transformation of maize with a nucleotide sequence of the disclosure (e.g., SEQ ID NO: 1 or SEQ ID NO: 3), the method of Zhao can be used (U.S. Pat. No. 5,981,840 and PCT patent publication WO98/ 32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of  $_{25}$ Agrobacterium under conditions whereby the bacteria are capable of transferring the nucleotide sequence (SEQ ID NO: 1 or SEO ID NO: 3) to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos can be immersed in an Agro- 30 bacterium suspension for the initiation of inoculation. The embryos are co-cultured for a time with the Agrobacterium (step 2: the co-cultivation step). The immature embryos can be cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" 35 step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of Agrobacterium without the addition of a selective agent for plant transformants (step 3: resting step). The immature embryos can be cultured on solid 40 medium with antibiotic, but without a selecting agent, for elimination of Agrobacterium and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). The 45 immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and calli grown on selective medium can be cultured on solid medium to regenerate the 50 plants.

#### Example 7—Transformation of Soybean Embryos

Soybean embryos are bombarded with a plasmid containing a nucleotide sequence of the disclosure (e.g., SEQ ID NO: 1 or SEQ ID NO: 3) operably linked to a suitable promoter as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of an appropriate soybean cultivar are 60 cultured in the light or dark at 26° C. on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged 65 embryos, the suspensions are maintained as described below.

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Soybean embryogenic suspension cultures can be maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26° C. with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein, et al., (1987) Nature (London) 327:70-73, U.S. Pat. No. 4,945,050). A Du Pont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene that can be used to facilitate soybean transformation includes, but is not limited to: the 35S promoter from Cauliflower Mosaic Virus (Odell, et al., (1985) Nature 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz, et al., (1983) Gene 25:179-188), and the 3' region of the nopaline synthase gene from the T DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising a toxin nucleotide sequence (e.g., SEQ ID NO: 1) operably linked to a suitable promoter can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50  $\mu$ L of a 60 mg/mL 1  $\mu$ m gold particle suspension is added (in order): 5  $\mu$ DNA (1  $\mu$ g/ $\mu$ L), 20  $\mu$ L spermidine (0.1M), and 50  $\mu$ L CaCl2) (2.5M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400  $\mu$ L 70% ethanol and resuspended in 40  $\mu$ L of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60×15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment the liquid media may be exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

All publications, patents and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this disclosure pertains. All publications, patents and patent applications are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing disclosure has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the embodiments.

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Asp	Leu	Thr 195	Arg	Leu	Ile	Pro	Ile 200	Tyr	Thr	Asp	Tyr	Ala 205	Val	Arg	Trp
Tyr	Asn 210	Thr	Gly	Leu	Aap	Arg 215	Leu	Pro	Arg	Thr	Gly 220	Gly	Leu	Arg	Asn
Trp 225	Ala	Arg	Phe	Asn	Gln 230	Phe	Arg	Arg	Glu	Leu 235	Thr	Ile	Ser	Val	Leu 240
Asp	Ile	Ile	Ser	Phe 245	Phe	Arg	Asn	Tyr	Asp 250	Ser	Arg	Leu	Tyr	Pro 255	Ile
Pro	Thr	Ser	Ser 260	Gln	Leu	Thr	Arg	Glu 265	Val	Tyr	Thr	Asp	Pro 270	Val	Ile
Asn	Ile	Thr 275	Asp	Tyr	Arg	Val	Gly 280	Pro	Ser	Phe	Glu	Asn 285	Ile	Glu	Asn
Ser	Ala 290	Ile	Arg	Ser	Pro	His 295	Leu	Met	Asp	Phe	Leu 300	Asn	Asn	Leu	Thr
Ile 305	Asp	Thr	Asp	Leu	Ile 310	Arg	Gly	Val	His	Tyr 315	Trp	Ala	Gly	His	Arg 320
Val	Thr	Ser	His	Phe 325	Thr	Gly	Ser	Ser	Gln 330	Val	Ile	Thr	Thr	Pro 335	Gln
Tyr	Gly	Ile	Thr 340	Ala	Asn	Ala	Glu	Pro 345	Arg	Arg	Thr	Ile	Ala 350	Pro	Ser
Thr	Phe	Pro 355	Gly	Leu	Asn	Leu	Phe 360	Tyr	Arg	Thr	Leu	Ser 365	Asn	Pro	Phe
Phe	Arg 370	Arg	Ser	Glu	Asn	Ile 375	Thr	Pro	Thr	Leu	Gly 380	Ile	Asn	Val	Val
Gln 385	Gly	Val	Gly	Phe	Ile 390	Gln	Pro	Asn	Asn	Ala 395	Glu	Val	Leu	Tyr	Arg 400
Ser	Arg	Gly	Thr	Val 405	Asp	Ser	Leu	Asn	Glu 410	Leu	Pro	Ile	Asp	Gly 415	Glu
Asn	Ser	Leu	Val 420	Gly	Tyr	Ser	His	Arg 425	Leu	Ser	His	Val	Thr 430	Leu	Thr
Arg	Ser	Leu 435	Tyr	Asn	Thr	Asn	Ile 440	Thr	Ser	Leu	Pro	Thr 445	Phe	Val	Trp
Thr	His 450	His	Ser	Ala	Thr	Asn 455	Thr	Asn	Thr	Ile	Asn 460	Pro	Asp	Ile	Ile
Thr 465	Gln	Ile	Pro	Leu	Val 470	Lys	Gly	Phe	Arg	Leu 475	Gly	Gly	Gly	Thr	Ser 480
Val	Ile	Lys	Gly	Pro 485	Gly	Phe	Thr	Gly	Gly 490	Asp	Ile	Leu	Arg	Arg 495	Asn
Thr	Ile	Gly	Glu 500	Phe	Val	Ser	Leu	Gln 505	Val	Asn	Ile	Asn	Ser 510	Pro	Ile

Thr	Gln	Arg 515	Tyr	Arg	Leu	Arg	Phe 520	Arg	Tyr	Ala	Ser	Ser 525	Arg	Asp	Ala
Arg	Ile 530	Thr	Val	Ala	Ile	Gly 535	Gly	Gln	Ile	Arg	Val 540	Asp	Met	Thr	Leu
Glu 545	Lys	Thr	Met	Glu	Ile 550	Gly	Glu	Ser	Leu	Thr 555	Ser	Arg	Thr	Phe	Ser 560
Tyr	Thr	Asn	Phe	Ser 565	Asn	Pro	Phe	Ser	Phe 570	Arg	Ala	Asn	Pro	Asp 575	Ile
Ile	Arg	Ile	Ala 580	Glu	Glu	Leu	Pro	Ile 585	Arg	Gly	Gly	Glu	Leu 590	Tyr	Ile
Asp	Lys	Ile 595	Glu	Leu	Ile	Leu	Ala 600	Asp	Ala	Thr	Phe	Glu 605	Glu	Glu	Tyr
Asp	Leu 610	Glu	Arg	Ala	Gln	Lys 615	Ala	Val	Asn	Ala	Leu 620	Phe	Thr	Ser	Thr
Asn 625	Gln	Leu	Gly	Leu	630	Thr	Asp	Val	Thr	Asp 635	Tyr	His	Ile	Asp	Gln 640
Val	Ser	Asn	Leu	Val 645	Glu	Cys	Leu	Ser	Asp 650	Glu	Phe	Cys	Leu	Asp 655	Glu
Lys	Arg	Glu	Leu 660	Ser	Glu	Lys	Val	Lys 665	His	Ala	Lys	Arg	Leu 670	Ser	Asp
Glu	Arg	Asn 675	Leu	Leu	Gln	Asp	Pro 680	Asn	Phe	Arg	Gly	Ile 685	Asn	Arg	Gln
Pro	Asp 690	Arg	Gly	Trp	Arg	Gly 695	Ser	Thr	Asp	Ile	Thr 700	Ile	Gln	Gly	Gly
Asp 705	Asp	Val	Phe	Lys	Glu 710	Asn	Tyr	Val	Thr	Leu 715	Pro	Gly	Thr	Phe	Asp 720
Glu	Cys	Tyr	Pro	Thr 725	Tyr	Leu	Tyr	Gln	Lys 730	Ile	Asp	Glu	Ser	Lys 735	Leu
Lys	Ala	Tyr	Thr 740	Arg	Tyr	Glu	Leu	Arg 745	Gly	Tyr	Ile	Glu	Asp 750	Ser	Gln
Asp	Leu	Glu 755	Ile	Tyr	Leu	Ile	Arg 760	Tyr	Asn	Ala	Lys	His 765	Glu	Thr	Val
Asn	Val 770	Pro	Gly	Thr	Gly	Ser 775	Leu	Trp	Pro	Leu	Ser 780	Ala	Gln	Ser	Pro
Ile 785	Gly	Lys	Cys	Gly	Glu 790	Pro	Asn	Arg	Сув	Ala 795	Pro	His	Leu	Glu	Trp 800
Asn	Pro	Asn	Leu	Asp 805	Сув	Ser	Сув	Arg	Asp 810		Glu	Lys	Сув	Ala 815	His
His	Ser	His	His 820	Phe	Ser	Leu	Asp	Ile 825	Asp	Val	Gly	Cys	Thr 830	Asp	Leu
Asn	Glu	Asp 835	Leu	Gly	Val	Trp	Val 840	Ile	Phe	ГÀа	Ile	Lys 845	Thr	Gln	Asp
Gly	Tyr 850	Ala	Arg	Leu	Gly	Asn 855	Leu	Glu	Phe	Leu	Glu 860	Glu	Lys	Pro	Leu
Leu 865	Gly	Glu	Ala	Leu	Ala 870	Arg	Val	Lys	Arg	Ala 875	Glu	ГÀа	Lys	Trp	Arg 880
Asp	Lys	Сув	Glu	Lys 885	Leu	Glu	Trp	Glu	Thr 890	Asn	Ile	Val	Tyr	Lys 895	Glu
Ala	Lys	Glu	Ser 900	Val	Asp	Ala	Leu	Phe 905	Val	Asn	Ser	Gln	Tyr 910	Asp	Arg
Leu	Gln	Ala 915	Asp	Thr	Asn	Ile	Ala 920	Met	Ile	His	Ala	Ala 925	Asp	Lys	Arg
Val	His	Ser	Ile	Arg	Glu	Ala	Tyr	Leu	Pro	Glu	Leu	Ser	Val	Ile	Pro

											- COI	ntin	nued	l				
	930					935				9,	40							
Gly 945	Val	Asn	Ala	Ala	Ile 950	Phe G	Glu G	lu Le	eu G: 95		ly Aro	g Il	e Phe	960				
Ala	Phe	Ser	Leu	Tyr 965	Asp	Ala A	Arg A		al II 70	le L	ys Ası	n Gl	y As <u>r</u> 975					
Asn	Asn	Gly	Leu 980	Ser	Cys	Trp A		al Ly 85	/s Gi	Ly H	is Val	99	•	Glu				
Glu	Gln	Asn 995	Asn	His	Arg		/al :	Leu V	/al V	/al 1		Lu '	Trp (	Blu Ala				
Glu	Val 1010		Gl:	n Glu	ı Val	. Arg 1015		CAa	Pro	Gly	Arg 1020	Gly	Tyr	Ile				
Leu	Arg 1025		l Th	r Ala	а Туг	1030		Gly	Tyr	Gly	Glu 1035	Gly	CÀa	Val				
Thr	Ile 1040		G Gl	u Ile	e Glu	1045		Thr	Asp	Glu	Leu 1050	ГÀа	Phe	Ser				
Asn	Сув 1055		l Gl	u Glu	ı Glu	1060	_	Pro	Asn	Asn	Thr 1065	Val	Thr	Cys				
Asn	Asn 1070	-	Th:	r Ala	a Thr	Gln 1075		Glu	His	Glu	Gly 1080	Thr	Tyr	Thr				
Ser	Arg 1085		n Ar	g Gl	7 Tyr	1090		Ala	Tyr	Glu	Ser 1095	Asn	Ser	Ser				
Val	His 1100		a Se	r Val	l Tyr	Glu 1105		Lys	Ser	Tyr	Thr 1110	Asp	Arg	Arg				
Arg	Glu 1115		n Pro	o Cys	s Glu	Ser 1120		Arg	Gly	Tyr	Gly 1125	Asp	Tyr	Thr				
Pro	Leu 1130		Al.	a Gly	/ Tyr	Val 1135		rys	Glu	Leu	Glu 1140	Tyr	Phe	Pro				
Glu	Thr 1145	-	ь Гу	s Val	l Trp	) Ile 1150		Ile	Gly	Glu	Thr 1155	Glu	Gly	Thr				
Phe	Ile	Va]	L As	p Sei	. Val	Glu	Leu	Leu	Leu	Met	Glu	Glu						

That which is claimed:

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1. A chimeric Cry1E polypeptide comprising: a) a Domain I of a first Cry1E protein; and b) a Domain II and a Domain III of a second Cry1E protein of SEQ ID NO: 2 45 or a fragment or variant of SEQ ID NO: 2, wherein the chimeric Cry1E polypeptide has at least 95% sequence identity to SEQ ID NO: 4.

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- 2. The chimeric Cry1E polypeptide of claim 1, wherein the first Cry1E protein is the polypeptide of SEQ ID NO: 6. 50
- 3. The chimeric Cry1E polypeptide of claim 1, wherein the Domain 1 has at least 95% sequence identity to amino acids 36-253 of SEQ ID NO: 6.
- **4**. The chimeric Cry1E polypeptide of claim **1**, wherein the Domain 1 comprises amino acids 36-253 of SEQ ID NO: 55
- **5**. The chimeric Cry1E polypeptide of claim **1**, wherein Domain 1 comprises amino acids 1-259 of SEQ ID NO: 6.
- **6**. The chimeric Cry1E polypeptide of claim **1**, where the Domain II and Domain III have at least 95% sequence 60 identity to amino acids 259-617 of SEQ ID NO: 2.
- 7. The chimeric Cry1E polypeptide of claim **5**, where the Domain II and Domain III comprise amino acids 259-617 of SEQ ID NO: 2.
- **8**. The chimeric Cry1E polypeptide of claim **1**, wherein 65 the chimeric Cry1E polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

- **9**. The chimeric Cry1E polypeptide of claim **1**, wherein the chimeric Cry1E polypeptide further comprises a Cry protoxin region.
- 10. The chimeric Cry1E polypeptide of claim 1, wherein the chimeric Cry1E polypeptide further comprises a heterologous signal sequence or a transit sequence.
- 11. A recombinant polynucleotide encoding the chimeric Cry1E polypeptide of claim 1.
- 12. The recombinant polynucleotide of claim 10 wherein said polynucleotide is a synthetic sequence that has been designed for expression in a plant.
- 13. The recombinant polynucleotide of claim 10 operably linked to a heterologous regulatory element.
- 14. A DNA construct comprising the recombinant polynucleotide of claim 10.
  - 15. A host cell comprising the DNA construct of claim 13.
- 16. The host cell of claim 14, wherein host cell is a bacterial cell.
- 17. The host cell of claim 14, wherein host cell is a plant cell.
- **18**. A transgenic plant comprising the DNA construct of claim **13**.
- 19. The transgenic plant of claim 17, wherein said plant is selected from the group consisting of maize, sorghum, wheat, cabbage, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugar beet, sugarcane, tobacco, barley, and oilseed rape.

- 20. A transformed seed of the plant of claim 17, wherein the seed comprises the DNA construct.
- **21**. An agricultural composition comprising the polypeptide of claim **1**.
- 22. The composition of claim 20, wherein said composition is in the form of a powder, dust, pellet, granule, spray, emulsion, colloid, or solution.
- 23. The composition of claim 20, comprising from 1% to 99% by weight of said polypeptide.
- **24**. A method for controlling a lepidopteran pest population, the method comprising contacting said population with a pesticidally-effective amount of the polypeptide of claim **1**.
- 25. A method for killing a lepidopteran pest, the method comprising contacting said pest with, or feeding to said pest, a pesticidally-effective amount of the polypeptide of claim 1. 15
- 26. A method for protecting a plant from a lepidopteran pest, the method comprising expression of the polynucle-otide of claim 10 in the plant.

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